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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project is to use phage antibody libraries to identify novel breast tumor antigens. The antibodies could be used for breast cancer immunotherapy and the antigens could be used as cancer vaccines. In the first year, we used a model system to identify the factors allowing successful phage antibody library selection on tumor cell lines. Multivalent display of phage antibodies led to more efficient selection of cell binding antibodies, as did recovery of phage from within the cell after binding to an internalizing cell surface receptor. The methods were used to select a panel of phage antibodies which bound the breast tumor cell line SKBR3. Some of the antibodies bound ErbB2, some the transferrin receptor, and one a novel antigen overexpressed on breast tumor cells. All were efficiently endocytosed as native antibody fragments and thus potentially useful for targeted cancer therapy. To widen the utility of this approach, a large human phage antibody library was constructed in a true phage vector in which multiple copies of antibody fragment are displayed on each phage. We have validated the utility of this library and are currently in the process of characterizing a large panel of breast tumor cell specific antibodies. We also developed a high throughput assay which allows rapid screening of unpurified antibody fragments for endocytosis into tumor cells.

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5. Introduction

A major goal of cancer research has been to identify tumor antigens which are qualitatively or quantitatively different from normal cells (1). The presence of such antigens could be detected by monoclonal antibodies that would form the basis of diagnostic and prognostic tests. In addition, the antibodies could be used to selectively kill tumor cells either directly via their effector function (2) or by attaching cytotoxic molecules to the antibody (3, 4).

Despite the demonstration of antigens which are overexpressed on tumor cells, antibodies have been used with limited success for diagnosis and treatment of solid tumors, (reviewed in ref. (5, 6)). Their utility has been hampered by the paucity of tumor specific antibodies, immunogenicity, low affinity, and poor tumor penetration. For this project, we proposed using a novel technology, termed phage display, to produce a new generation of antibodies which would overcome the limitations of previously produced anti-tumor antibodies (ref. 7-11). The antibodies would bind breast cancer antigens with high affinity, be entirely human in sequence, and would penetrate tumors better than IgG.

5.1. Purpose of the present work and methods of approach

For this work, we proposed to isolate and characterize a large assortment of high affinity human and murine antibody fragments that bound to specific breast cancer antigens and to normal antigens that are overexpressed on cancer cells. Antibodies isolated using phage display would be used for early sensitive diagnosis of node-negative breast cancer patients, for immunotherapy prior to growth of large tumor mass, and as adjuvant therapy for minimal residual disease. Human antibodies were to be isolated from a very large and diverse phage antibody library of >6,700,000,000 different members (12). Murine antibodies would be isolated from libraries constructed from the B-lymphocytes of mice immunized with breast tumor cell lines. Antibodies that recognize antigens which were overexpressed or unique to breast carcinomas would be isolated by selection on breast tumor cell lines and characterized with respect to affinity and specificity.

The proposed technical objectives in the statement of work were:

Task 1: Create phage antibody libraries from mice immunized with malignant breast tissue and with the tumor cell lines MDA MB231, ZR-75-1 and SKBR3 (months 1-18).

a. Immunize mice with appropriate cell line or tissue.
b. Prepare mRNA, amplify V_H and V_L genes, create scFv gene repertoires.
c. Construct phage antibody libraries.

Task 2: Create subtractive phage antibody libraries from mice immunized with malignant breast tissue and with the tumor cell lines MDA MB231, ZR-75-1 and SKBR3 (months 1-18).

a. Immunize mice with appropriate normal cell line or tissue and deplete repertoire with Cytoxan.
b. Immunize mice with the appropriate tumor cell line or tissue.
c. Prepare mRNA, amplify V_H and V_L genes, create scFv gene repertoires.
d. Construct phage antibody libraries.

Task 3: Isolate and characterize scFv antibody fragments which bind novel breast tumor antigens by selecting phage antibody libraries on malignant breast tissue, parafin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3. (months 12-30).

a. Determine optimal conditions for selecting cell surface binding phage antibodies using C6.5 ErbB2 binding phage antibody, an irrelevant hapten binding phage antibody and ErbB2 expressing SK-OV-3 cells.
b. Determine optimal conditions for selecting internalizing phage antibodies using C6.5 ErbB2 binding phage antibody and SKBR3 cells.

c. Select a 6.7 x 10^9 member human phage antibody library on malignant breast tissue, parafin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3.

d. Select phage antibody libraries constructed in tasks 1 and 2 on malignant breast tissue, parafin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3.

e. Determine scFv specificity on a panel of cell lines and tissues.

Task 4: Determine the antigens recognized by tumor specific scFv using Western blotting, immunoaffinity purification, and protein sequencing. (months 24-36).

a. Create scFv affinity columns for antigen immunopurification.

b. Identify tumor specific antigens by Western blotting, immunoaffinity purification and protein sequencing.

c. Create subtractive tumor cell line phage cDNA library.

d. Select tumor cell phage cDNA library on purified monoclonal scFv which recognize tumor specific antigens.

6. Body of report

When we proposed and submitted this project in early 1997, it was unclear as to the likelihood of obtaining tumor cell specific antibodies by selecting phage antibody libraries directly on tumor cell lines. While our group was the first to report the successful selection of cell binding antibodies from phage libraries by direct selection on erythrocytes (13), reports of subsequent successful cell selections have been interspersed with reports of failures. Thus we proposed using both large non-immune phage antibody libraries and libraries constructed from mice immunized with tumor cell lines. The advantage of large non-immune phage libraries is that they can be constructed from human variable region genes and thus yield human antibodies, ideal for use as therapeutics. The advantage of using murine libraries is that at least in theory the libraries can be enriched for antibodies which bind the immunizing cell line.

Since the construction of phage antibody libraries is a difficult and time consuming task, we focused during the first year of funding on determining the factors affecting the successful selection of cell binding phage antibodies using a model system (Task 3a and 3b). Results of this work have been reported in prior progress reports and also published (14). Results from these studies indicated that: 1) phage antibodies can be endocytosed in a receptor dependent manner; and 2) that enrichment ratios were in the range where direct selection from a library should be possible. Factors leading to optimal selection were also identified.

Once the optimal method of selection was determined, proof of concept was demonstrated using a non-immune human phage antibody library (Task 3c). This work followed on work begun in the latter year of funding from DAMD17-94-J-4433 and continued throughout this project.

6.1 Selection and characterization of cell binding and internalizing antibodies from a phage antibody library

A non-immune phagemid antibody library was utilized to identify new phage antibodies that were bound and were internalized into SKBR3 cells (Task 3c). A selection strategy illustrated in Nielsen & Marks, ref 15 and figure 4, appendix 1 was utilized. After 3 rounds of selection, 40% of randomly picked clones bound SKBR3 cells and of these, 50% bound ErbB2 by ELISA (Table 2, Poul et. al. Ref 16 and appendix 2). This is not surprising, given that SKBR3 cells express very high levels of ErbB2. DNA fingerprinting indicated that 2 unique antibodies (F5 and C1) binding ErbB2 were obtained. These stained ErbB2 expressing cell lines comparably to other ErbB2 antibodies and in proportion to the cell lines known level of ErbB2 expression (Table 3, Poul et. al., appendix 2 and ref. 16). Many additional antibodies were obtained that did not bind ErbB2 and that preferentially bound tumor cell lines but not normal

cell lines (Table 3, Poul et. al., appendix 2). One of these antibodies which stained a number of tumor cell lines (H7) was studied further. The H7 gene was subcloned into a secretion vector and native hexahistidine tagged scFv was purified and used to immunoprecipitate the antigen it recognized from an SKBR3 cell lysate. After excision from a gel and protein sequencing, the antigen recognized by H7 was determined to be the transferrin receptor. F5 stained ErbB2 in Western blot (figure 2, Poul et. al., appendix 2), and both F5 and H7 could immunoprecipitate their respective antigens from SKBR3 cell lysates (figure 2, Poul et. al., appendix 2). As either phage antibodies or native monomeric scFv antibody fragment, both F5 and H7 were efficiently endocytosed by SKBR3 cells (figure 3 and 4, Poul et. al., appendix 2). In the case of H7 (but not F5) endocytosis served as a surrogate marker for growth inhibition, with H7 exhibiting dose dependent inhibition of the growth of SKBR3 cells (figure 5, Poul et. al., appendix 2). H7 competed with holotransferrin for binding to the transferrin receptor, explaining probably both the mechanism of growth inhibition and the mechanism by which it was able to induce receptor mediated endocytosis of the transferrin receptor (figure 6, Poul et. al., appendix 2).

In the current year, additional antibodies from the phagemid library selected on SKBR3 cells have been characterized with respect to tumor cell specificity and antigen recognized. Recently, we have extensively characterized an additional phage antibody obtained from selections on SKBR3 cells that has an interesting pattern of immunoreactivity. The antigen identification process developed was as proposed for Task 4. The antibody, S5, stains the tumor cell lines SKBR3 and MCF7 intensely by flow cytometry, stains the transformed cell lines MCF10A less intensely, and only slightly stains the tumor cell lines MDA231 (figure 1, page 10). To identify the antigen recognized by the scFv, we subcloned the scFv gene into a vector which fuses a C-terminal hexahistidine tag for purification. ScFv was expressed, harvested from the bacterial periplasm and purified by immobilized metal affinity chromatography as previously described. Staining of the appropriate cell lines by the native scFv was confirmed by flow cytometry. A number of techniques were explored to determine the optimal means of antigen identification by immunoprecipitation. To verify that immunoprecipitated antigen was indeed from the cell surface, we lightly biotinylated the surface of SKBR3 cells with NHSS biotin. This allows precise visualization of immunoprecipitated proteins by SDS-PAGE followed by Western blotting and detection with streptavidin-HRP. Two techniques were explored for immunoprecipitation: 1) immunoprecipitation using Ni-NTA agarose, which takes advantage of the universal hexahistidine tag on the scFv; and 2) immunoprecipitation with Protein A. This particular scFv is derived from the human VH3 family and thus binds Protein A. After biotinylation of the cell surface, antigen was immunoprecipitated from SKBR3 cell lysates, run on a SDS-PAGE gel and Western blotted with antigen detection using streptavidin-HRP. After immunoprecipitation with Protein A, a single dominant band was seen on Western blot (figure 2, page 10). When the corresponding acrylamide gel was visualized with Comassie blue, a single band was also visualized in the area stained in Western blot (data not shown). This band was excised and sent for liquid chromatography and tandem mass spectrometry. After immunoprecipitation with Ni-NTA agarose, several bands were visualized on Western blot (figure 2, page 10), one of these was of the same apparent molecular mass as the band immunoprecipitated using Protein A. In contrast to immunoprecipitation with Protein A, many bands were visualized when the corresponding acrylamide gel was visualized with Comassie blue. The large number of bands present precluded excision of the band for sequencing. Multiple conditions were explored to reduce the number of proteins immunoprecipitated by Ni-NTA, but none were found that gave reduced the number of proteins immunoprecipiated. Based on SDS-PAGE and Western blotting to identify surface proteins, it appears that the majority of these additional bands represent intracellular proteins that have histidines in the proper orientation to bind Ni-NTA.

Immunoprecipitated antigen was identified by tryptic fragmentation of the excised protein, followed by liquid chromatography to separate peptides and tandem mass spectrometry to identify peptide sequence. 10 of 11 peptides matched to the protein encoded by the kiaa gene, with the sequences spanning the protein sequence (figure 2, page 10) (17). The

protein contains a putative cell attachment sequence (RGD), a transmembrane domain, and 6 Ig like domains (http://www.kazusa.or.jp/huge/). Probing of the SAGE database indicates that the gene is most highly expressed in 3 breast and 2 ovary cell lines. The function of this gene product is unknown, but it interacts with CD9 and other tetraspanins and may be involved in cell migration/metastasis (18). To verify that the scFv actually recognized the kiaa1436 gene product, CHO cell transfected with the gene were obtained from Charrin (18). The S5 scFv stained kiaa1436 transfected cells but did not stain untransfected CHO cells (figure 3, page 11).

The results demonstrate that tumor specific phage antibodies can be directly selected from phage libraries by panning on tumor cell lines and recovering phage which have triggered receptor mediated endocytosis from within the cytosol. Such antibodies are efficiently endocytosed by the target cell line, both as phage antibodies and as native scFv antibody fragments. As such, these antibodies are likely to be ideal for delivery of drugs or genes into the cytosol for therapeutic application. For example, during the current year, we have developed the F5 ErbB2 scFv as a targeting antibody for doxorubicin containing immunoliposomes (IL). F5 was developed under funding from DAMD, and preclinical work on IL funded largely by our Breast Cancer SPORE. F5 ErbB2 scFv have been inserted into liposomes containing doxorubicin to create IL. In preclinical models, F5-IL cause a significantly greater reduction in tumor growth than untargeted IL (see Nielsen et al, appendix 3 and ref. 19). Based on extensive preclinical results, F5 scFv expression was scaled at the NCI-MARP for GMP manufacture for a phase 1 clinical trial. Within the last month, F5 scFv and F5-IL have been inlicensed by Alza-Johnson and Johnson to complete preclincal work and initiate a phase 1 clinical trial estimated to begin April 2003.

In some instances (as with H7), endocytosis can be used as a surrogate marker for direct desirable biologic effects exhibited by the antibody, in this case growth inhibition

Significance: We have developed methodology and protocols which allows direct selection of tumor specific antibodies from a phage library on the basis of their ability to trigger receptor mediated endocytosis. We have used this approach to generate internalizing antibodies to ErbB2 and have developed a therapeutic drug (ErbB2 immunoliposomes) based on one of these antibodies that is being manufactured at the NCI MARP and which will enter clinical trials. There is no more stringent validation of the quality of an antibody. The approach appears applicable to other tumor cell lines and generates antibodies to known and novel tumor antigens. A subset of the internalizing antibodies will have direct tumor cell inhibitory effects, validating that internalization can be used as a surrogate marker of growth inhibition, and probably apoptosis.

6.2 The selection approach is general and applicable to other cell lines

To show general applicability of this approach, and to generate Abs to the EGF receptor, we performed similar selections on A431 cells which overexpress EGFR and also on Chinese Hamster Ovary cells which overexpress EGFR. For both selections, 2 scFv Abs were obtained which bound EGFR expressing cells but did not bind cell lines which did not express EGFR (20). Representative results are shown in the figures in Heitner et al. , appendix 4 and ref. 20. While we have not yet determined whether these scFv have any direct cytotoxic effects, the results illustrate that this selection methodology can be applied to other tumor cell lines.

6.3 Generation of a non-immune phage antibody library in a true phage vector

Our results above indicate that cell surface selections are most efficient (give the highest enrichment ratios) when the scFv antibody fragment is displayed in multiple copies on the surface of bacteriophage. This occurs when the phage antibody library is constructed in a true phage vector containing all of the phage genome. To date, all non-immune and most immune phage antibody libraries have been constructed in phagemid vectors. The remainder of the phage genes and proteins are provided by infecting E. coli harboring the phagemid antibody with a helper phage. Since the helper phage provides wild type pIII, the majority of phage antibodies have only a single copy of scFv-pIII fusion protein, with the remaining 4 copies of

pIII being wild type. Libraries to date have been constructed in phagemid vectors for two reasons: 1) the transformation efficiencies are much higher, making it easier to construct large libraries; and 2) the concern that multivalent display may lead to selection of lower affinity phage antibodies due to avidity.

Our data indicates that even with very high affinity (1 nM) antigen binding, cell surface selection results in very low enrichment ratios, even when binding an internalizing receptor and recovering phage from within the cell. Thus construction of immune phagemid libraries (as proposed in tasks 1 and 2) did not make sense. Rather we chose to construct true phage libraries, as a new task to replace tasks 1 and 2. Given the technical difficulties in generating large phage libraries from cDNA, to validate the utility of phage libraries for generating antibodies binding cell surface antigens, we elected to construct a true phage antibody library by subcloning the scFv gene repertoire from our existing phagemid library (ref. 12) into a phage vector into which we have engineered compatible cloning sites for the scFv gene repertoire (fd-TET/Sfi-Not). This allows preparation of large quantities of phagemid vector harboring the scFv gene repertoire from which the scFv gene repertoire can be excised as Sfi-Not restriction enzyme fragments. fd-TET/Sfi-Not vector DNA was prepared by digestion with the same two restriction enzymes and the scFv gene repertoire ligated into vector DNA. After multiple transformations (> 50) a library containing 5.0×10^8 transformants was obtained. PCR screening of 20 randomly selected colonies indicated that 100% had a scFv sized insert and fingerprinting indicated that the library was diverse. In the current year, we characterized this library with respect to its ability to generate antigen specific antibodies and compared the number of antibodies and their binding constants with those obtained from our phagemid library. We have demonstrated that compared to phagemid libraries, true phage libraries generate a greater number of unique antibodies per target antigen (O'Connell et al, Tables 1 and 2, appendix 5 and ref. 21). We also demonstrated that the true phage library can be successfully selected on cells (fetal erythrocytes) to generate antibodies with exquisite specificity (see Huie et al. Ref 22 and appendix 6). We have selected this library on the breast tumor cell lines MCF7 and BT474 and are currently in the process of characterizing antibodies from these selections.

6.4 Generation of a high throughput assay for cell binding and endocytosis

One factor limiting our ability to identify recombinant phage antibodies which bind and internalize into tumor cells is a high throughput assay for cell binding and endocytosis. While we have used cell ELISA, it has a high background and only reports cell binding, not endocytosis. As we have been working with liposomes, a means occurred to us to generate a high throughput assay for endocytosis. Our scFv can easily be engineered to have a C-terminal hexahistidine tag. This can be achieved by batch subcloning the output scFv gene repertoire after each round of selection. We have been able to construct liposomes containing a fluorescent reporter dye and having on their surface Ni-NTA which has been inserted into the lipid coat of the liposome. We hypothesize that such liposomes should be able to chelate the hexahistidine tagged scFv (directly from the bacterial supernatant without the need for purification). If the scFv binds an internalizing epitope, the fluorescent liposome will enter the cell. ScFv and liposomes remaining on the cell surface can easily be removed by washing with EDTA. Cells are then lysed and if the scFv binds an internalizing epitope, a fluorescent signal will occur. Using Ni-NTA liposomes and the internalizing and non-internalizing scFv we have generated to date, we have validated this assay and determined its sensitivity. The assay is sensitive down to an scFv concentration of approximately 1ug/ml, a concentration easily obtainable from bacterial supernatants. The assay also is only positive when the scFv is internalizing (for example F5 scFv). We will utilize this assay to screen the selection results to identify additional internalizing antibodies.

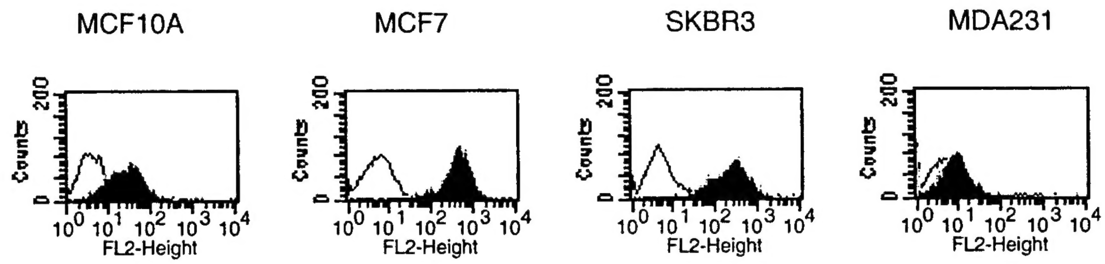

Figure 1. Staining of tumor cell lines MCF7, SKBR3, and MDA231 and transformed cell line MCF10A by the phage antibody S5. Phage binding was detected using anti-M13 antibody.

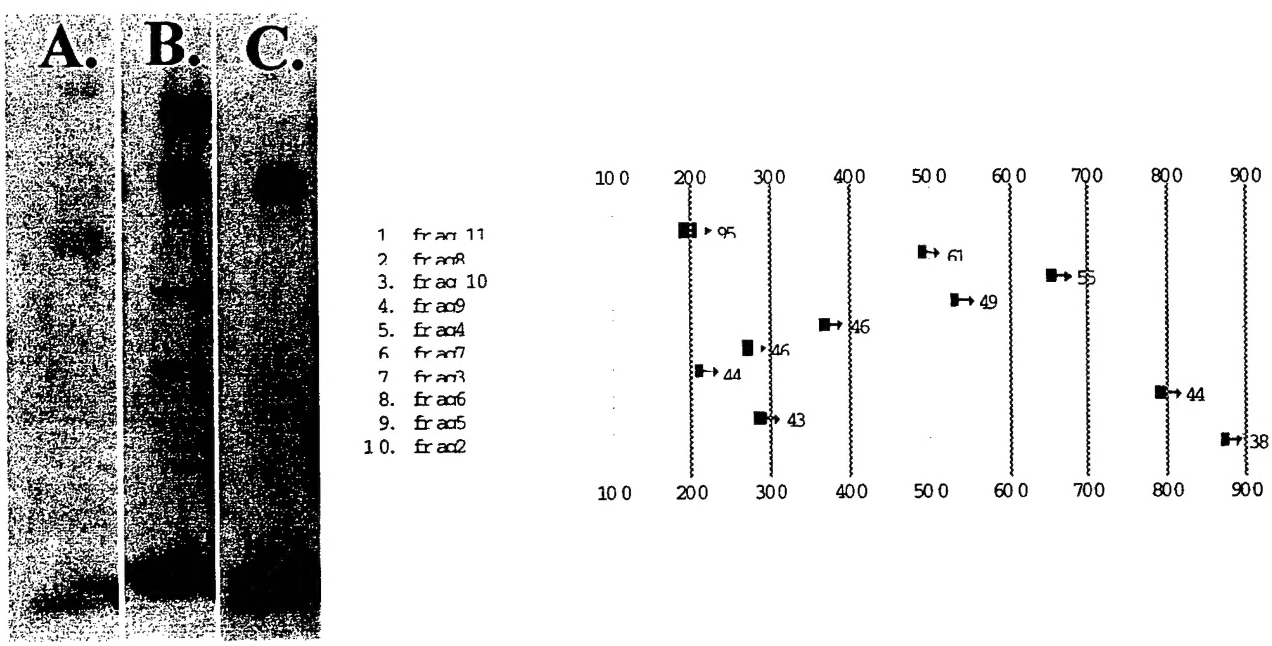

Figure 2. Western blot of proteins immunoprecipitated by the phage antibody S5 and identification by LC-tandem mass spectrometry. Left panel: SKBR3 cell surface proteins were biotinylated by incubation with NHSS biotin. Cells were then lysed and incuabted with S5 scFv. ScFv bound antigens were then immunoprecipitated by incubation with either Ni_TA agarose, lane B or Protein A, lane C. After washing, beads were loaded into the lanes of an polacrylamide gel, electrphoresed, transferred to nitrocellulose by blotting, and surface proteins detected by streptaviding-HRP. Lane A = control using Protein A immunoprecipitation without scFv. Right panel. Peptides sequenced by :C-tandem mass spec were used to screen the NCBI sequence database. 10 of 11 peptides matched portions for the KIAA1436 gene (AB037857), spanning the majority of the protein sequence (blue boxes=peptides.

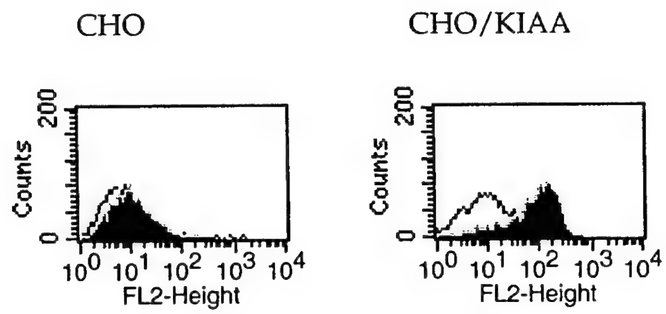

Figure 3. Flow cytometry analysis of S5 phage antibody binding to CHO cell transfected with the kiaa1436 gene and untransfected CHO cells (Control).

7. Key research accomplishments

- Identification of optimal phage antibody format for selection of phage antibodies on tumor cells
- Demonstration that phage antibodies binding cell surface receptors can trigger receptor mediated endocytosis
- Identification of optimal phage antibody format for selection of internalizing phage antibodies on tumor cells
- Successful selection of tumor specific phage antibodies from a non-immune phage antibody library, including ErbB2, transferrin receptor and the kiaa gene product
- Successful validation of the ErbB2 scFv F5 in preclinical models as capable of delivering immunoliposomes containing doxorubicin to tumors, with therapeutic effect
- Transfer of scFv F5-IL to a corporate partner (Alza-Johnson and Johnson) for completion of preclinical work and clinical trials
- Construction of a large non-immune phage antibody library in a true phage vector
- Validation of phage library as a source of scFv antibodies for purified antigens and cell surface receptors

8. Reportable outcomes

8.1 Nielsen, U.B. and Marks, J.D. Internalizing antibodies and targeted cancer therapy: direct selection from phage libraries. Pharmaceutical Sciences and Trends Today. 3: 282-291, 2000.

8.2 Poul, M.-A., Becerril, B., Nielsen, U.B., Morisson, P., and Marks, J.D. Selection of tumor-specific internalizing human antibodies from phage libraries. J. Mol. Biol. 301: 1149-1161, 2000.

8.3 Heitner, T., Moor, A., Garrison, J. L., Hasan, T., and Marks, J. D. (2001) J. immunol. Meth. Selection of cell binding and internalzing epidermal growth factor receptor antibodies from a phage display library. 248, 17-30.

8.4 Nielsen, U.B., Kirpotin, D.B., Pickering, E.M., Hong, K., Park, J.W., Shalaby, R., Shao, Y., Benz, C.C., and Marks, J.D. Biophys. Biochim. Acta. In press

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9. Conclusions

9.1. We have identified conditions which allow successful selection of cell binding and internalizing phage antibodies by panning phage libraries directly on cells (Tasks 3a and 3b).

9.2 We have demonstrated successful selection of tumor specific antibodies by panning a non-immune phage antibody library on the SKBR3 tumor cell line (Task 3c).

9.3 During the current year, we characterized additional internalizing phage antibodies from this selection with respect to specificity and antigen recognized. We also developed a novel method for antigen identification using phage antibodies to immunoprecipitate biotinylated surface antigens followed ny LC-tandem MS.

9.4. We have constructed and characterized true multivalent phage libraries as leading to more efficient selection of antigen and cell binding and internalizing antibodies.

9.5 We developed a high throughput assay to identify cell binding and internalizing antibodies after selection.

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11. Appendices

Internalizing antibodies and targeted cancer therapy: direct selection from phage display libraries

Ulrik B. Nielsen and James D. Marks

Antibody internalization is required for the success of many targeted therapeutics, such as immunotoxins, immunoliposomes, antibody–drug conjugates and for the targeted delivery of genes or viral DNA into cells. Recently, it has become possible to directly select antibody fragments from phage display libraries for internalization into mammalian cells. Here we review the therapeutic applications of internalized antibodies and describe how phage display enables the isolation of internalizing antibodies to novel or known targets.

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▼ Although antibodies show tremendous promise for the treatment of human malignancies, initial attempts to develop anti-tumor antibodies were generally unsuccessful. These failures were largely caused by the limitations of murine hybridoma technology including, for example, the immunogenicity of murine antibodies in humans. More recently, both improved understanding of tumor biology and advances in antibody engineering have made it possible to identify better tumor targets for antibody-based therapies and to generate less immunogenic humanized and human antibodies.

Studies of the molecular basis of tumorigenesis have identified cell surface receptors that are either: (1) tumor or lineage specific, such as CD20 (Ref. 1) and mutant forms of the epidermal growth factor receptor[2] (EGF receptor; see Glossary in Box 1), or (2) overexpressed in tumors, such as ErbB2 (Ref. 3). These cell surface receptors serve as ideal antibody targets.

Technologies for making antibodies

Advances in molecular cloning and antibody engineering have made it possible to convert rodent monoclonal antibodies into chimeric antibodies (where the constant regions are human) or humanized antibodies (where the majority of the variable region sequence is also human). Such antibodies, especially those that are humanized, are significantly less immunogenic than rodent antibodies and can be consecutively admini-stered without an increase in clearance or loss of efficacy.

Human antibodies

Several technologies have recently been developed to produce antibodies of entirely human origin. Transgenic mice harboring a portion of the human variable region (V) gene locus have enabled human antibodies to be produced using standard hybridoma technology[4]. Although this approach generates antibodies of entirely human sequence, it has similar limitations to traditional hybridoma technology. It relies on the availability of an immunogen and a natural immune response, and may yield only a limited number of anti bodies, often directed to a few immunodominant

Box 1. Glossary

EGF Epidermal growth factor
Ig Immunoglobulin
V-gene Immunoglobulin variable region gene
scFv Single chain Fv antibody fragment
PEG Polyethylene glycol
IL Immunoliposomes
PE *Pseudomonas* exotoxin
RAIT Radioimmunotherapy
NCAM Neural cell adhesion molecule
PSMA Prostate-specific membrane antigen
CEA Carcinoembryonic antigen

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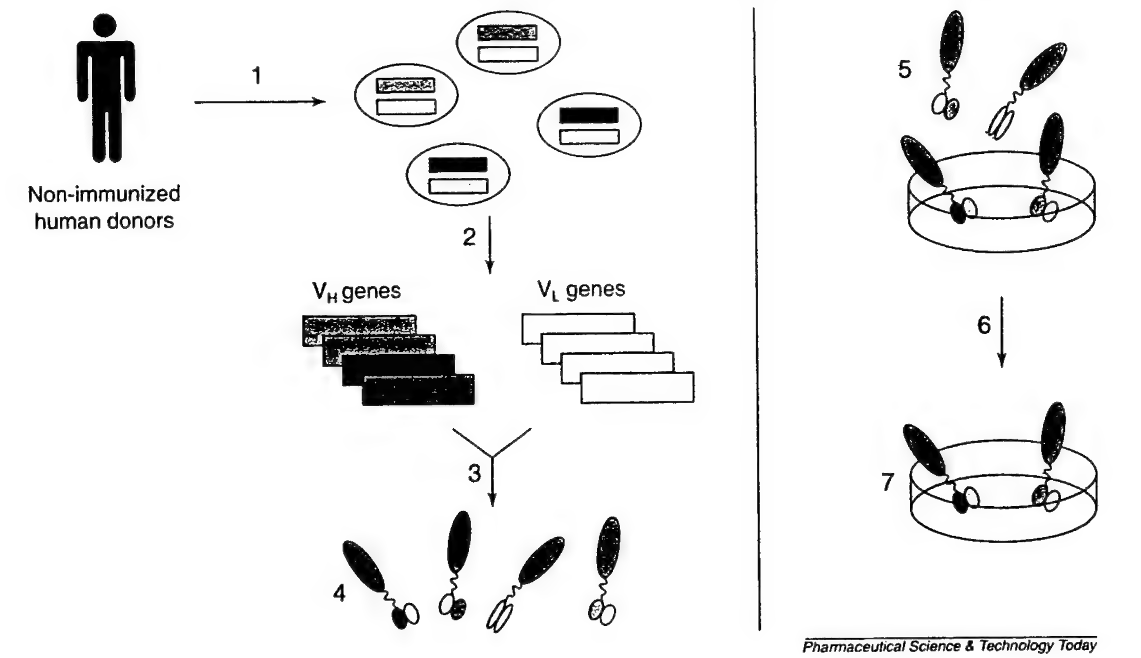

Figure 1. Schematic diagram depicting the cloning and selection of naïve phage display antibody libraries: (1) B-lymphocytes obtained from peripheral blood or spleen provide a source of naïve V-genes for repertoire construction; (2) the V-genes are amplified by PCR using V-region-specific primers; (3) a splice overlap PCR reaction assembles the V_H and V_L genes with a peptide linker creating an scFv antibody gene repertoire; (4) the PCR products are cloned into a phage display vector in frame with the gene encoding the pIII phage capsid protein resulting in phage displaying the antibody library; (5) antigen-binding phage antibodies are selected by binding the phage to antigen-coated plates; (6) washing away phage that do not bind antigen; and (7) eluting antigen-bound phage with strong acid or base and re-infecting *Escherichia coli* to prduce more phage for additional rounds of selection.

epitopes. Phage display is another promising technology, which has produced antibody fragments that bind a wide variety of antigens, including several hitherto refractory immunogens[5–7]. Combinatorial antibody libraries are typically cloned from naïve repertoires of immunoglobulin (Ig) V-genes, such as IgM genes, from non-immunized donors and are displayed on phage (Fig. 1).

Gene fragments encoding the Ig heavy and light chain variable regions (V_H and V_L) are amplified from B-lymphocytes using PCR and are assembled as single-chain Fv antibody fragments (scFv). The assembled genes are inserted into a phage display vector in frame with the gene encoding the phage coat protein pIII. Following its introduction into *Escherichia coli*, the random combinatorial library of antibody fragments is displayed on phage. Antigen-specific antibodies can be selected from antibody libraries displayed on phage after one week and the antibody fragments typically express at high levels in *E. coli*[8].

Another advantage of phage display is that the antibody genes are directly available for the subsequent genetic engineering of the antibody fragment, that is, to make fusion molecules[9] or to improve antibody affinity[10]. The genetic engineering of antibody fragments has also enabled an extensive study of the physical properties of antibodies affecting the targeting of human malignancies. Several parameters such as affinity[11], valence[12,13], charge[14] and size[15] have previously been shown to influence tumor targeting *in vivo*.

Antibody strategies for cancer therapy

As a result of the advances in antibody engineering and tumor biology, the first two antibodies approved for therapy of human cancers entered clinical practice: (1) Rituxan for non-Hodgkins lymphoma[16] and (2) Herceptin for breast cancer[17]. These two antibodies were developed on the basis of their ability to bind cell surface receptors overexpressed on the target tumor (CD20 in the case of non-Hodgkins lymphoma and ErbB2 in breast cancer). Rituxan and Herceptin exert their therapeutic effects directly, either by inducing apoptosis (Rituxan[18,19]) or by causing growth inhibition (Herceptin[20]). Only a fraction of the antibodies generated against a known surface receptor, such as ErbB2, share this direct tumor inhibitory ability[21]. If the antibodies do not directly inhibit tumor growth, other strategies using the antibody to deliver a toxic payload must be used.

Many of these strategies rely on the ability of the antibody to bind to the surface receptor in a manner that induces

receptor-mediated endocytosis, resulting in the delivery of the cytotoxic agent into the cytosol. For example, anti-ErbB2 antibodies have been used to target doxorubicin-containing liposomes[22] or *Pseudomonas* exotoxin (immunotoxin) in the interior of tumor cells[9,23]. The use of antibodies to target non-viral gene therapy vectors also requires the antibody to induce receptor-mediated endocytosis in order to deliver the gene into the cell. Similar to growth inhibition, the majority of antibodies generated by immunization do not bind to receptors in a manner that triggers endocytosis[21,24], and it is therefore essential to select for antibodies that can elicit the desired response.

Exploiting receptor-mediated endocytosis for drug delivery

The endocytic pathway can be used by antibodies to deliver drugs into the cytosol. Typically, endocytosis plays a role in numerous cellular functions including antigen presentation, nutrient acquisition, receptor regulation and synaptic transmission. Endocytic pathways are also used by viruses, toxins and symbiotic microorganisms to gain entry into cells.

Internalization via clathrin-coated pits

One of the most well-characterized endocytic mechanisms is receptor-mediated endocytosis via clathrin-coated pits. The binding of ligands to receptors often leads to receptor aggregation, either by inducing a conformational change or by cross-linking receptors[25,26]. In the case of the EGF receptor[27] and the Fc receptor type II (Ref. 28), the receptors subsequently concentrate in clathrin-coated pits resulting in endocytosis and clearance from the cell surface. Membrane proteins that are internalized in clathrin-coated pits contain targeting sequences in their cytoplasmic domains that interact with a variety of adaptor proteins, and clathrin, which directs the protein into these pits[29]. The fate of the receptor–ligand complex after it is in the endocytic vesicle is dependent on the receptor. For example, the transferrin receptor enters the early endosomes from which it is rapidly recycled along with transferrin to the cell surface[30]. By contrast, the EGF receptor is either recycled following dissociation of EGF or it accumulates in the late endosomes wherein it is degraded.

Drug delivery via internalizing antibodies

Antibodies and antibody fragments can deliver a variety of agents, such as drugs, genes, toxins and radionuclides, to target cells that express the antigen. The endocytosis of the antibody fragment to the interior of the cell can often increase the effect of the therapeutic agent. A major advantage of receptor-mediated endocytosis as a drug delivery route is that therapeutic agents can be delivered specifically into target cells that overexpress the receptor and thereby increase efficacy while reducing systemic toxicity. The main disadvantage is that the therapeutic agent localizes to the endosomes, but it needs to escape from here into the cytoplasm in order to exert its pharmacological effects.

Immunoconjugates

Monoclonal antibodies directed to tumor-associated antigens have been chemically conjugated to a variety of drugs such as doxorubicin[31] and more-toxic molecules such as enediynes[32]. Most immunoconjugates rely on the release of the drug from the antibody after it is in the endosome in order for it to exert its pharmacological activity in the cytosol or nucleus. Immunoconjugates that are internalized into cells by receptor-mediated endocytosis enter endosomes and lysosomes that contain a mildly acidic (pH 4–5) environment. This pathway offers a selective mechanism of drug release if drug carrier linkers have adequate differences in their rates of hydrolysis at lysosomal and systemic pH. Alternatively, the release of the drug from the antibody following internalization can take advantage of the metabolic potential of the endosomes and lysosomes[33].

Targeted gene delivery

To accomplish antibody-mediated gene delivery, the antibody must contain a domain that will complex or encapsulate the DNA vector. This can be a non-specific carrier domain, such as protamine[34], or natural protein domains that bind specific DNA sequences[35]. Whatever the carrier, after targeting to the cell surface the DNA must enter the cell nucleus for gene expression. Receptor-mediated endocytosis has been investigated as a pathway for non-viral gene delivery into cancer cells; however, after it has entered the endosome, the gene must be released from the carrier and must enter the cytosol.

Research into how viruses escape from endosomes has resulted in the enhancement of gene expression using membrane-active peptides derived from viral domains[36], and translocation to the nucleus has been improved using nuclear localization signals[37]. Also, cationic lipid–DNA complexes that efficiently escape the endosomes have been targeted to tumors. Such agents, however, are rapidly cleared from the circulation. Thus, the highest levels of activity are observed in 'first pass' organs, such as the lungs, spleen and liver. Viruses, which inherently escape from the endosome, have also been targeted with antibodies. Engineered viruses, however, can generate an immune response that can compromise transfection efficiency on subsequent injections. In addition, the natural wild-type tropism must be attenuated to obtain tumor target specificity. Eventually, a better understanding of endosomal escape will lead to targeted gene delivery constructs that achieve high gene expression without the potentially harmful toxicity associated with viral gene delivery.

Immunoliposomes

Several liposomal drugs, such as the liposome-encapsulated doxorubicin, have proven to be effective against cancer in clinical trials[38]. The steric stabilization of liposomes with polymers such as polyethylene glycol (PEG) have increased the circulation time by reducing the rate of reticuloendothelial clearance and increasing the uptake by tumors[39]. The coupling of antibodies to liposomes to form immunoliposomes (ILs) shows promise for increasing the efficacy of liposomal drugs against solid tumors and leukemia by specific interaction with the tumor cells. In early studies, a strong association between enhanced growth inhibition and liposome internalization was observed *in vitro*[40]; however, the binding of ILs displaying antibodies is not always followed by internalization[41]. Since these studies, numerous investigations have demonstrated that the cytotoxicity of the liposome-encapsulated drug increases when the liposome carrier is internalized into the target cell[40,42–45]. Enhanced efficacy *in vivo* also appears to depend on internalization. When the monoclonal anti-ErbB2 antibody N-12A5 was coupled to sterically stabilized liposomes, no increased efficacy over untargeted liposomes was observed[41]. By contrast, when an internalizing anti-ErbB2 Fab was used for IL construction, greatly enhanced efficacy in a mouse xenograft model[46] was observed owing to enhanced IL uptake into tumor cells (D. Kirpotin *et al.*, unpublished).

Immunotoxins

Immunotoxins are attractive candidates for cancer therapy because they combine the specificity of tumor-cell-reactive antibodies with the high cytotoxic potency of naturally occurring toxins[9,23]. *Pseudomonas* exotoxin (PE) is frequently used for immunotoxin construction. PE and related toxins consist of three regions involved in binding, translocation and activity. The translocation domain is believed to actively transport the active domain from the endosome into the cytosol. This makes toxins such as PE well suited for targeting by receptor-mediated endocytosis because this is the pathway that the toxin naturally transits before entering the cytosol, where it efficiently inhibits protein synthesis. Indeed, immunotoxins have shown efficacy in several clinical trials, particularly for the treatment of hematological tumors[47]. Endocytosis of the antigen–immunotoxin complex appears to be the most important determinant of *in vitro* cytotoxicity. Other factors, such as the extent of cell binding and the number of cell surface antigens, appear to affect cytotoxicity only to the degree that they influence endocytosis[48].

Radionuclide antibody conjugates

Radioimmunotherapy (RAIT) or immunoscintigraphy using systemically administered antibodies linked to radionuclides is a promising approach to the treatment and diagnosis of cancer. It is not immediately obvious that antibody internalization is advantageous for RAIT and immunoscintigraphy. Following internalization, radioiodinated antibodies are usually degraded and dehalogenated intracellularly[49], leading to the conclusion that non-internalized antibodies would be superior. However, the intracellular degradation of radiolabelled antibodies and the subsequent secretion of radioactive iodine does not seem to prevent the accumulation of intracellular radioactivity. Indeed, the accumulation and retention of radioactivity in the tumor tissue, owing to the internalization of radiolabelled antibody, improved the immunoscintigraphy of xenografts in nude mice[50]. Furthermore, dehalogenation in the cell only takes place when iodine nuclides are attached to tyrosine residues using Trouts reagent[51] (other radionuclides such as ^{111}In or chelated nuclides can also be used). In RAIT, internalization of the antibodies used for targeting are also advantageous. The emission characteristics of the radioisotope are critical in determining the appropriate radiation dose to the tumor compared with normal organs. If antibodies internalize and transport low-energy electron-emitting isotopes close to the tumor cell nucleus, an improved therapeutic advantage can be achieved. In the case of Auger emitters such as ^{125}I, lower toxicity is observed. This is probably caused by the short path length of their low-energy electrons, which can reach the nuclear DNA only if the antibody is internalized[52].

For several other antibody-based strategies, internalization can be prohibitive. For instance, bi-specific antibodies and immune-stimulatory fusion proteins require interaction on the cell surface with cells of the immune system, thus making internalization undesirable. Likewise, internalization is not desired for antibody-directed enzyme prodrug therapy in which an antibody-bound enzyme is localized to the cell surface where it enzymatically converts a prodrug.

Factors that influence antibody internalization

Several approaches have been used to develop antibody-based delivery systems that use endocytosis as a point of entry into cells. The limitations of endocytosis as an entry point for drugs into cells depends on the: (1) type of receptor, (2) antigen density, (3) epitope, (4) rate of internalization, (5) release of the therapeutic molecule from the endosome, and (6) re-expression of the antigen on the cell surface (Fig. 2).

Several antibodies to cell surface receptors, such as the EGF receptor[53,54], ErbB2 (Refs 55,56) and transferrin receptor[57], induce internalization. Other cell surface molecules have also been shown to mediate antibody internalization (although often at a slower rate). These include the neural cell adhesion molecule[50] (NCAM), prostate-specific membrane antigen[58] (PSMA), carcinoembryonic antigen[59] (CEA) and mucins[60]. The

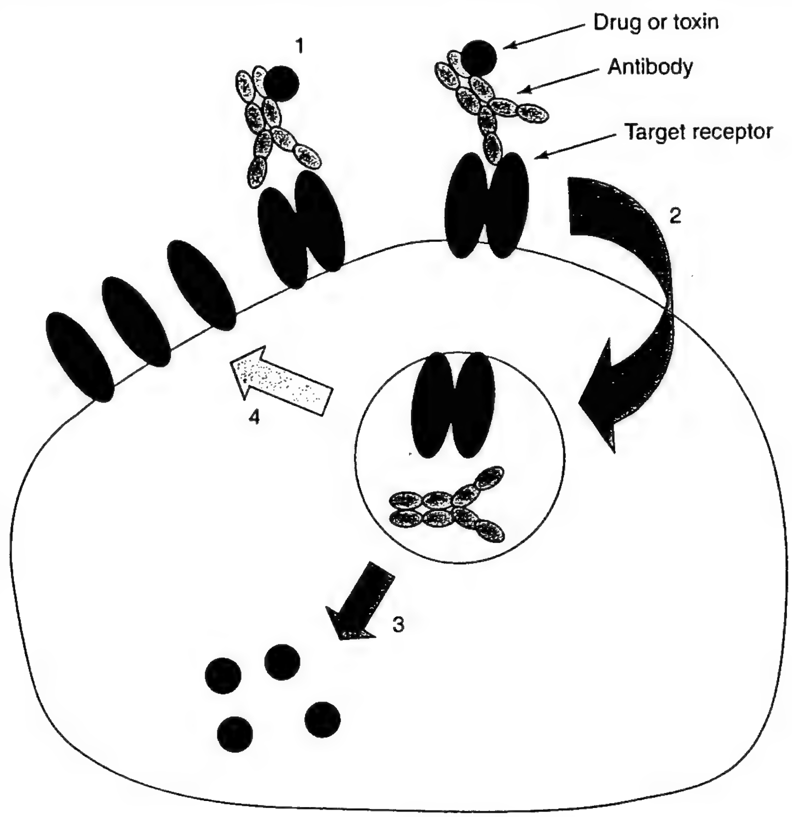

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Figure 2. Antibody targeting of drugs to cells via receptor-mediated endocytosis: (1) antibody conjugated to a therapeutic agent, such as a toxin, drug, radionuclide, liposome or DNA, binds to receptor overexpressed on the target cell; (2) antibody binding triggers internalization of the receptor; (3) typically, the therapeutic agent must escape from the endosome in order to exert its action in the cytoplasm or nucleus; and (4) the receptor is either recycled to the surface or degraded in the endosome.

therapeutic potential of antibodies and antibody-targeted drugs has been correlated with antigen density on the target cell surface[61,62]. A high density of cell surface receptors permits more antibodies to be concentrated on target cells, which consequently results in greater pharmacological effectiveness[62]. The choice of antigen is a key factor for targeting malignancies because, to a large extent, it determines the rate of internalization and intracellular routing[43,63]. Indeed, the rate of internalization plays a key role in predicting the cytotoxicity of drug or toxin conjugates[64,65].

However, not all antibodies binding to internalizing receptors are rapidly internalized[21,56]. It appears that the epitope recognized by the targeting antibody influences the rate of internalization[65,66]. Frequently, the antibody mimics the natural ligand. For instance, some internalizing antibodies against the EGF receptor cause tyrosine phosphorylation and, in some cases, also mimic the mitogenic effects of EGF[54,67]. Tyrosine phosphorylation of the EGF receptor, however, is not a requirement for antibody internalization[68]. Similar discrepancies have been reported for the activities of internalizing anti-ErbB2 monoclonal antibodies[56,69].

Most of the investigations carried out on the internalization of antibodies did not address the role of multi-valency in antibody internalization. For many antibodies, however, bivalency seems to be mandatory for internalization. When monovalent Fab fragments of several anti-ErbB2 antibodies were tested for internalization, the fragments were not internalized[70]. Similar observations were reported for the Fab portion of an anti-EGF receptor antibody[53]. Further, increasing the valency of antibodies can also increase their internalization. For instance, internalization of IgG aggregates by polymorphonuclear neutrophils varies with the size of the aggregates[71]. Thus, caution must be used when designing recombinant antibody targeted drugs with a monovalent binding site, such as scFv or Fab, to ensure efficient internalization.

Screening antibodies for internalization

The most common method for monitoring the internalization of ligands and antibodies into cells uses a low pH buffer (typically glycine-HCl, pH 2.8) to dissociate the surface-bound antibody. However, reports from several groups indicate that this buffer, in some cases, only partially dissociates antigen–antibody complexes and therefore can introduce major inaccuracies in internalization experiments[72,73]. Alternatively, antibodies can be biotinylated with NHS-SS-biotin and incubated with live cells. Following the specific reduction of biotin groups on cell-surface-bound antibodies with reducing agents, internalization can be quantitated by immunoblotting[58]. However, the accuracy of this method also relies on the complete removal of biotin from the cell-surface-bound antibody. All of the above-mentioned screening methods are laborious, allowing only a limited number of different antibodies to be screened for internalization.

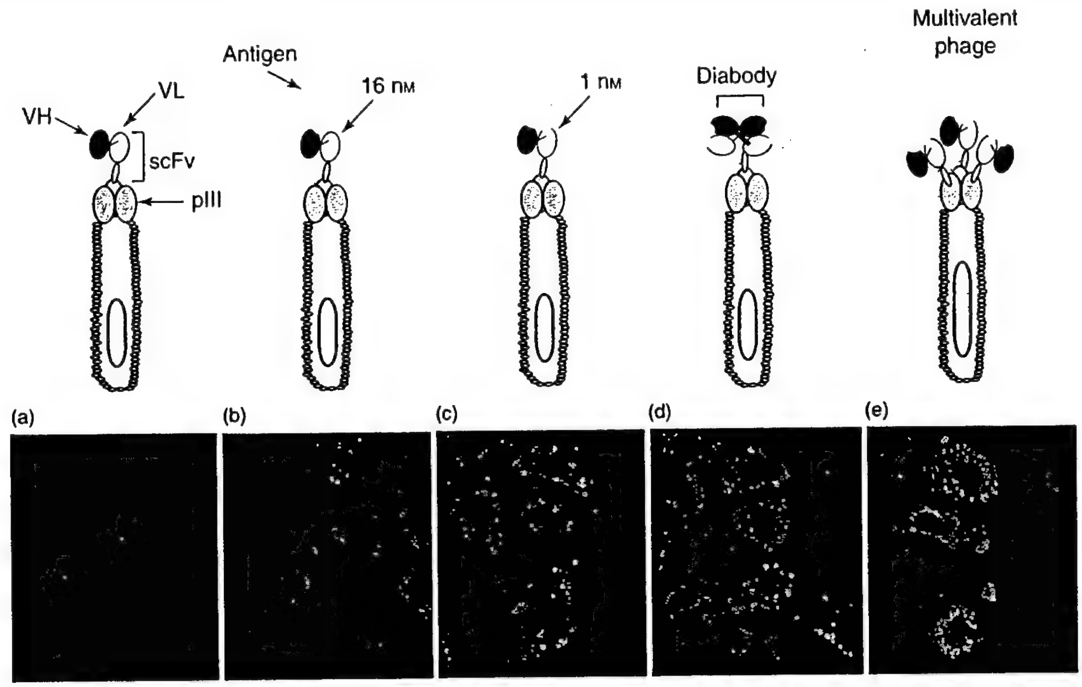

Figure 3. Internalization of anti-ErbB2 phage derivatives. The ErbB2-overexpressing cancer cell line SKBR3 was grown on coverslips and incubated with (a) control phage antibody; (b) phagemid displaying single copies of anti-ErbB2 scFv, K_D = 16 nM; (c) phagemid displaying single copies of anti-ErbB2 scFv K_D = 1 nM; (d) phage displaying single copies of anti-ErbB2 bivalent diabody; or (e) multivalent phage displaying 3–5 copies of anti-ErbB2 scFv for 2 hours at 37°C. Intracellular phage was detected with biotinylated anti-M13 antiserum and Texas-Red streptavidin. (Adapted from Ref. 80.)

Direct selection of internalizing antibodies from phage display libraries

Because phage antibody isolation takes place *in vitro*, selection procedures can be manipulated to select for antibodies with desired physical or biological activities. Recently, the direct selection of peptides and antibody fragments binding cell surface receptors from filamentous phage libraries by the incubation of phage libraries with a target cell line has been demonstrated[6,74–77]. This has led to an increase in the number of potential targeting molecules. However, the isolation of cell-type-specific antibodies from naïve libraries has been difficult because selections often result in the isolation of antibodies to common cell-surface antigens[78].

Phage internalization into mammalian cells

The ability of bacteriophage displaying peptides to undergo receptor-mediated endocytosis[75,79] indicates that phage antibody libraries might be selected not only for their cell binding but also for their internalization into mammalian cells. Unlike peptide phage libraries, however, phage antibody libraries typically display monomeric scFv or Fab antibodies fused to pIII as single copies on the phage surface (phagemid libraries). It has been hypothesized that such monovalent display is unlikely to lead to efficient receptor cross-linking and phage internalization. To determine the feasibility of selecting internalizing antibodies and to identify the factors responsible for phage internalization, the C6.5 phage antibody has been investigated. C6.5 scFv binds the growth factor receptor ErbB2, which is overexpressed in many solid tumors. Similar to the majority of antibodies, monovalent C6.5 scFv is only minimally internalized, although the bivalent diabody is efficiently endocytosed[80]. Investigations have also been carried out on the phagemid displaying single copies of C6.5 scFv[81], phagemid displaying a 16-fold higher affinity mutant of C6.5 scFv[10], phagemid displaying single copies of the bivalent C6.5 diabody[12] and phage displaying multiple copies of C6.5 (Fig. 3). For these studies, the phage were incubated with live cells at 37°C, the surface-bound phage was removed by acid washing and the endocytosed phage recovered by cell lysis.

The internalization of monovalent C6.5 scFv was only fourfold greater than the internalization of a non-specific anti-botulinum phage antibody (background internalization).

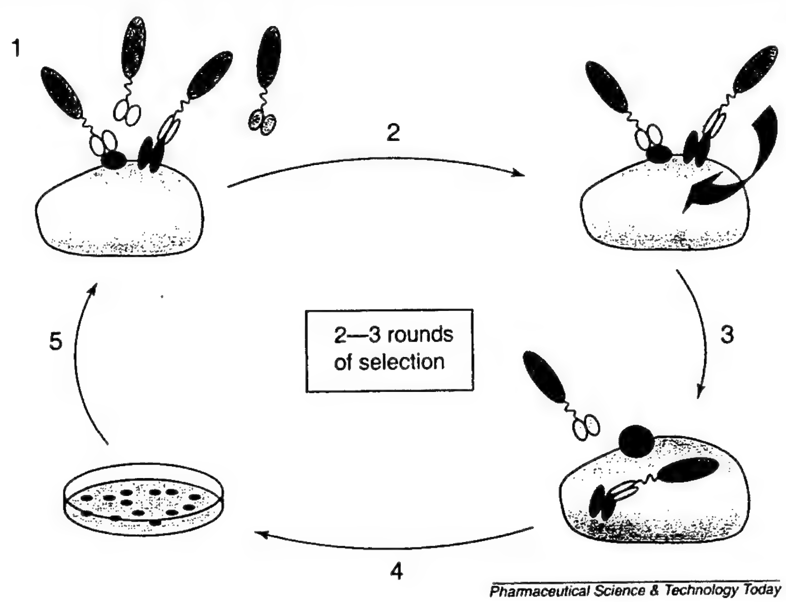

Figure 4. Selection for antibody internalization from a phage display library: (1) phage antibody library is incubated with the target cells at 4°C to reduce internalization; (2) unbound phage is washed away and cells are returned to 37°C for < 15 minutes to enable the internalization of phage bound to internalizing receptors; (3) cell-surface-bound phage are stripped with a low pH acid buffer; (4) cells are lysed and phage recovered by reinfection into *Escherichia coli*; and (5) phage are re-amplified for additional rounds of selection.

Display of the 16-fold higher affinity C6.5 mutant increased internalization to 16-fold above background. Endocytosis was greatest when the phage antibody was multivalent, prepared either by using the bivalent diabody or by display on a phage vector. The uptake of multivalent phage increased to 30-fold (diabody) and 146-fold (phage vector) above background. For any display format, the enrichment ratio above background was much greater for internalized phage compared with phage recovered from the cell surface (only two-fold to 20-fold above background). The result of the uptake of the different phage antibodies as determined by fluorescence microscopy agreed with uptake determined by phage titering (Fig. 3). These experiments demonstrated the feasibility of directly selecting internalizing antibodies from phage libraries and indicated that selection is most efficient for bivalent diabodies or scFv displayed multivalently on phage.

Selection of internalizing antibodies from phage display libraries

The strategy described above was used to directly select from a large naïve phage antibody library[7] a scFv capable of triggering endocytosis into breast tumor cells upon receptor binding[82] (Fig. 4). This library displays single copies of monovalent scFv using a phagemid vector. Although our results described above indicate that this is not the optimal display format for internalization selections, no diabody or phage based naïve libraries currently exist.

After three rounds of selection, greater than 50% of the scFv analysed bound to the selecting cell line. The further characterization of several of these antibodies identified two that bound ErbB-2 (F5 and C1) and three that bound the transferrin receptor. Interestingly, both ErbB-2 and the transferrin receptor are rapidly internalized and are specific markers for several cancers[83]. Both the phage antibodies and the native purified scFv were rapidly endocytosed into cells expressing the appropriate receptor. The scFv that bind ErbB2 and the transferrin receptor did not spontaneously dimerize and do not require dimerization in order to undergo internalization[82]. The internalization of the anti-ErbB2 scFv F5 was compared with the C6.5 scFv, which was selected on recombinant ErbB2 protein using traditional panning strategies[81]. The antibodies recognize different epitopes on ErbB2 with comparable affinities; however, C6.5 scFv does not significantly internalize into ErbB2 as monomeric scFv, whereas F5 scFv does.

Using this strategy of selection for internalization into tumor cell lines, internalizing antibodies to ErbB2, transferrin receptor and EGF receptor have now been isolated. In addition, hundreds of antibodies to unidentified targets have been isolated, several of which appear to be overexpressed on breast cancer cells. Given the nature of the antigens identified to date, the identification of novel antigens from these selections using expression cloning and protein microsequencing are currently being pursued. It is envisaged that selection on other cell types will identify other cell-specific markers because endocytosed receptors are likely to be associated with specific cell functions, such as growth factor and nutrient transport receptors on cancer cells or Fc receptors on cells of the immune system. This approach can also be used to generate internalizing antibodies to known receptors by transfecting the receptor into an appropriate cell line and performing selections as described above. It might also be possible to use internalization as a surrogate marker to identify desirable biological effects of the antibody, for example, apoptosis or growth inhibition. Indeed,

a significant growth inhibitory effect of the anti-transferrin scFv identified in the selection on cancer cells was observed[82]. Thus, antibodies selected using this approach might have a direct therapeutic effect in addition to the ability to deliver drugs into the cytosol.

Human scFv antibodies selected from a phage display library for internalization into tumor cells can readily be used as targeting molecules for drug delivery. For example, one of the scFv against ErbB2 was conjugated to the surface of commercial liposomal doxorubicin converting it into fully functional doxorubicin-loaded anti-ErbB2 immunoliposomes (U.B. Nielsen *et al.*, unpublished). The resulting ILs have superior efficacy in an ErbB2 overexpressing mouse xenograft model compared with untargeted liposomal doxorubicin. Because of the entirely human origin of the scFv, it is likely that the resulting ILs will be non-immunogenic in humans.

Conclusion

It is envisaged that the selection for internalization methodology will be generally applicable to generate scFv capable of delivering liposomes or other agents into a wide variety of tumor cells, such as prostate and ovarian cancers. In addition, the generation of multivalently displayed antibody fragment libraries (either as diabodies or on phage vectors) should greatly increase the number of internalizing antibodies generated using this approach. Compared with scFv isolated from monovalently displayed libraries, these antibody fragments might need to be used multivalently (as on liposomes) in order to be internalized. Alternatively, they might be useful as monovalent scFv to target the cell surface for bispecific therapeutic approaches because they are not likely to be endocytosed in that format.

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Approval...

Schering–Plough (Madison, NJ, USA) have announced the recommendation of approval by the EU's Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) for the use of CAELYX (pegylated liposomal doxorubicin hydrochloride) in the treatment of advanced ovarian cancer. It is proposed that CAELYX will be administered intravenously once every four weeks for as long as the disease does not progress and the treatment is tolerated by the patient.

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Selection of Tumor-Specific Internalizing Human Antibodies from Phage Libraries

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Antibody internalization into the cell is required for many targeted therapeutics, such as immunotoxins, immunoliposomes, antibody-drug conjugates and for targeted delivery of genes or viral DNA into cells. To generate directly tumor-specific internalizing antibodies, a non-immune single chain Fv (scFv) phage antibody library was selected on the breast tumor cell line SKBR3. Internalized phage were recovered from within the cell and used for the next round of selection. After three rounds of selection, 40% of clones analyzed bound SKBR3 and other tumor cells but did not bind normal human cells. Of the internalizing scFv identified, two (F5 and C1) were identified as binding to ErbB2, and one (H7) to the transferrin receptor. Both F5 and H7 scFv were efficiently endocytosed into SKBR3 cells, both as phage antibodies and as native monomeric scFv. Both antibodies were able to induce additional functional effects besides triggering endocytosis: F5 scFv induces downstream signaling through the ErbB2 receptor and H7 prevents transferrin binding to the transferrin receptor and inhibits cell growth. The results demonstrate the feasibility of selecting internalizing receptor-specific antibodies directly from phage libraries by panning on cells. Such antibodies can be used to target a variety of molecules into the cell to achieve a therapeutic effect. Furthermore, in some instances endocytosis serves as a surrogate marker for other therapeutic biologic effects, such as growth inhibition. Thus, a subset of selected antibodies will have a direct therapeutic effect.

Keywords: receptor mediated endocytosis; ErbB2; phage antibody library; single chain Fv; tumor targeting

Introduction

Antibody internalization into the cell is required for many targeted therapeutics, such as immunotoxins (Altenschmidt *et al.*, 1997), immunoliposomes (Kirpotin *et al.*, 1997), antibody-drug conjugates and for targeted delivery of genes or viral DNA into cells (Fominaya & Wels, 1996). This can be accomplished by taking advantage of normal receptor biology: ligand binding causes receptor activation *via* homo- or heterodimerization, either directly for a bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor-mediated endocytosis (Ullrich & Schlessinger, 1990). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cell. In general, this requires a bivalent antibody capable of mediating receptor dimerization (Heldin, 1995; Yarden, 1990). In addition, the effi-

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Abbreviations used: scFv, single chain Fv; cfu, colony forming units; EGFR, epidermal growth factor receptor; TEA, triethylamine; ELISA, enzyme linked immunosorbent assay; ECD, extracellular domain; CHO, Chinese hamster ovary cells; IMAC, immobilized metal affinity chromatography; HRP, horseradish peroxidase; TfR, transferrin receptor; V_H, immunoglobulin heavy chain variable domain; V_L, immunoglobulin light chain variable domain; FBS, fetal bovine serum; FCS, fetal calf serum; EGF, epidermal growth factor; RT, room temperature; BSA, bovine serum albumin; FACS, fluorescent activated cell scanning; HBS, Hepes-buffered saline.

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ciency with which antibodies mediate internalization differs significantly depending on the epitope recognized (Hurwitz *et al.*, 1995; Yarden, 1990).

Currently, antibodies which trigger internalization are identified by screening antibodies derived by either hybridoma or phage antibody technology. However, this usually involves examining antibodies recognizing specific targets, and while it may take the biology of the target into account, it takes no account of the biology of the antibody that is triggering receptor endocytosis. Antibodies which trigger receptor endocytosis could be directly selected from large non-immune phage libraries (Marks *et al.*, 1991; Sheets *et al.*, 1998) by recovering infectious phage particles from within cells after receptor-mediated endocytosis, as reported for peptide phage libraries (Hart *et al.*, 1994; Barry *et al.*, 1996). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (de Haard *et al.*, 1999; Knappik *et al.*, 2000; Marks *et al.*, 1991; Nissim *et al.*, 1994; Sblatero & Bradbury, 2000; Sheets *et al.*, 1998; Vaughan *et al.*, 1996). To determine the feasibility of selecting internalizing antibodies, we previously studied the human scFv C6.5 which binds the growth factor receptor ErbB2 (Schier *et al.*, 1995). Using wild-type C6.5 scFv displayed monovalently in a phagemid system, we demonstrated that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis, albeit with very low efficiency and enrichment ratios (Becerril *et al.*, 1999). The low efficiency reflects the expectation that a monomeric binding unit is unable to mediate receptor cross-linking and phage internalization. Since C6.5 was selected for binding to native protein, we reasoned that if scFv were able to mediate internalization in the monomeric form, the most effective way to identify them would be by direct selection from a large non-immune phage library by recovery of infectious phage particles from within tumor cells. Here we report the successful selection and characterization of such tumor-specific internalizing antibodies.

Results

Selection of internalizing phage antibodies

For selections, phage were prepared from a 7.0×10^9 member human scFv phage antibody library (Sheets *et al.*, 1998). To select for internalizing phage antibodies, 5×10^6 subconfluent adherent SKBR3 breast tumor cells were incubated with 1×10^{12} colony forming units (cfu) of phage in the presence of normal human fibroblasts in suspension for 1.5 hours. This step was performed at 4 °C to allow phage binding without internalization. The fibroblasts were used to deplete the library of phage antibodies which bound to antigens common to SKBR3 cells and fibroblasts. Two different sets of selections were performed in parallel. For one set of selections, fibroblast depletion was performed during each round of selection. For the second set of selections there was no fibroblast depletion in the first round to avoid the potential loss of rare phage antibodies which bound to antigens that were quantitatively but not qualitatively different between the selecting and depleting cell lines. After phage binding, the cells were washed extensively with phosphate-buffered saline (PBS) to remove non-specifically or weakly bound phage. Cells were then incubated at 37 °C for 15 minutes to allow endocytosis of surface-bound phage. Fifteen minutes was chosen, since it is long enough to allow ligand-induced internalization of receptors like epidermal growth factor receptor (EGFR) and ErbB2 and short enough to avoid phage degradation within the cell, which would impair the recovery of infectious phage (Becerril *et al.*, 1999). To recover phage from within the cell, the cells were stripped three times with a low pH glycine buffer to remove phage bound to the cell surface, trypsinized and washed with PBS to remove phage bound in the extracellular matrix or to the culture plate, and finally lysed with high pH triethylamine (TEA). The cell lysate containing phage recovered from within the cell was used to infect *Escherichia. coli* TG1 to prepare phage for the next round of selection. A total of three rounds of selection were performed. Selections were monitored by titering: (1) the number of phage bound to the cell surface in the first low pH glycine wash (wash 1); and (2) the number of endocytosed phage recovered from within the cell (cell lysate) (Table 1). For both selection strategies (±depletion on fibroblasts in the first round), the titer of phage bound to the cell surface increased only four- to tenfold after three rounds of selection while the titer of phage recovered in the cell lysate increased 100 to 200-fold (Table 1, and data not shown for selection without depletion in round 1). This resulted in more than a 100-fold increase in the number of endocytosed phage recovered per cell, from 0.01 phage/cell up to 3.75 phage/cell (Table 1). These data suggest that phage were selected on the basis of endocytosis into SKBR3 cells.

Initial characterization of phage antibodies

Antibodies binding SKBR3 cells were identified by cell ELISA using native soluble scFv expressed from randomly picked single colonies from the second and third rounds of selection. When depletion was included in each round of selection, 11/94 (11.7%) of the clones bound SKBR3 cells after two rounds of selection and 55/135 (40.7%) bound SKBR3 cells after three rounds of selection (Table 2). All positive clones gave no signal above background on fibroblasts in a cell ELISA assay, indicating that the library had been efficiently depleted of antibodies common to fibroblasts and SKBR3. The frequency of SKBR3 ELISA positive clones was similar when depletion was not

Table 1. Results of phage antibody library selection on SKBR3 cells

Round	Number of fibroblasts used for depletion	Phage input	Phage output: Wash 1 (cell surface)	Phage output: Cell lysate	Phage input/output ratios ($\times 10^{-8}$): Wash 1 (cell surface)	Phage input/output ratios ($\times 10^{-8}$): Cell lysate	Phage output/cell: Number of cells	Phage output/cell: Number of phage/cell (cell surface)	Phage output/cell: Number of phage/cell (intracellular)
1	0	3.0E + 12	3.6E + 06	9.7E + 04	120	3.2	ND	ND	ND
2	5.0E + 06	2.7E + 12	1.1E + 06	5.0E + 04	40	1.8	3.4E + 06	0.32	0.01
3	4.5E + 06	8.4E + 12	4.4E + 07	1.0E + 07	523	119	3.9E + 06	11.3	2.7
1	5.0E + 06	3.0E + 12	3.8E + 06	3.5E + 04	126	1.2	2.7E + 06	1.4	0.01
2	4.5E + 06	1.0E + 13	2.0E + 06	1.2E + 05	20	1.2	3.3E + 06	0.6	0.04
3	1.2E + 06	1.7E + 13	1.3E + 07	7.5E + 06	76	44	2.0E + 06	6.5	3.8

ND, not determined.

included in the first round; however, the majority of these antibodies (greater than 90%) also bound fibroblasts (data not shown). Thus, depletion during each round of selection was essential for the selection of cell-specific antibodies. Subsequent characterization focused only on the library which had been depleted during each round of selection.

Since SKBR3 cells are known to express high levels of the internalizing receptor ErbB2, bacterial supernatants containing soluble scFv were screened for binding to ErbB2 extracellular domain (ECD) by ELISA. After three rounds of selection, 29/135 clones (21%) bound ErbB2 ECD (Table 2). This represents approximately 50% of the antibodies which bound SKBR3 cells. The number of unique ErbB2 binders was determined by PCR fingerprinting of the 29 ELISA positive clones followed by DNA sequencing. Two unique ErbB2-binding human scFv (F5 and C1) were identified. Neither of these antibodies was similar in sequence to the 14 anti-ErbB2 scFv obtained selecting the same phage antibody library on purified recombinant ErbB2 ECD (Sheets *et al.*, 1998).

Further characterization of ErbB2 binding antibodies

F5 and C1 phage antibodies were analyzed for binding to a panel of cell lines whose ErbB2 expression had been described (Lewis *et al.*, 1993). They both stained SKBR3 and SKOV3 cells strongly, stained MCF7 cells weakly and did not stain fibroblasts and the normal breast cell line Hs578Bst (Table 3). The same profile was observed using phage displaying the C6.5 scFv that recognizes ErbB2 (Schier *et al.*, 1995) and the anti-ErbB2 monoclonal antibody 4D5 (Sarup *et al.*, 1991). F5 and C1 did not stain Chinese hamster ovary (CHO) cells or CHO cells transfected with the EGF receptor but did stain CHO cells transfected with ErbB2 (not shown). For subsequent studies, the F5 and C1 scFv genes were subcloned into a vector which fused a C-terminal hexahistidine sequence, expressed from *Escherichia coli* TG1 and purified by immobilized metal affinity chromatography (IMAC). Gel filtration analysis indicated that both F5 (Figure 1) and C1 existed exclusively as monomeric scFv with no apparent spontaneous dimerization or aggregation as seen with some scFv

Table 2. Frequency of binding clones after selection of a phage library on SKBR3 cells

Round of selection	ErbB2 positive phage (%)[a]	SKBR3 positive phage (%)[b]
1 (First round no depletion)	ND	ND
2	0.50	ND
3	1.10	ND
1 (First round depleted)	ND	ND
2	5.10	11.70
3	21.40	40.70

ND, not determined.

[a] As determined by ELISA of 96 random clones on recombinant ErbB2 ECD.

[b] As determined by ELISA of 96 random clones on SKBR3 cells.

Table 3. Characterization of phage antibody binding to a panel of cells by flow cytometry

Primary antibody	Cell type: SKBR3	MCF7	SKOV3	LNCaP	Fibroblasts
A. ErbB2 positive scFv-phage					
C6.5	526[a]	16	670	nd	1
F5	4867	123	5839	nd	11
C1	858	0	566	nd	0
4D5 (Mab)	600	29	586	nd	2
B. ErbB2 negative scFv-phage					
3TE3	1056	1002	416	nd	130
H7	4003	1219	945	nd	93
3TB5	225	301	336	199	9
2TF5	1973	495	805	nd	0
3TH8	153	1	353	1	0
3TG5	469	80	714	82	3
3TF12	611	83	31	233	7
2TB4	138	3	1	nd	1
C2-1	181	8	81	nd	1
3GD8	103	6	1154	45	0

nd, not determined.

[a] Results are expressed in mean fluorescent intensity (MFI) minus background fluorescence.

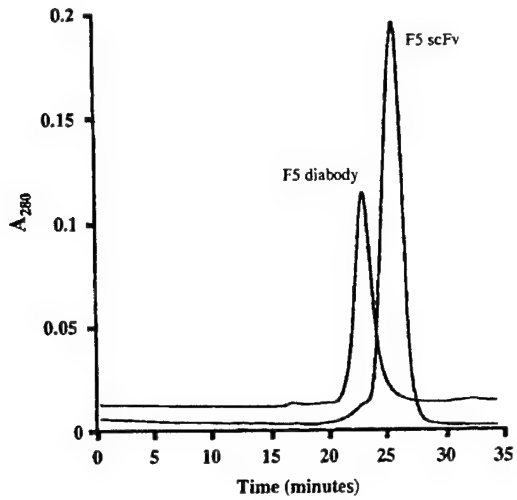

Figure 1. Gel filtration analysis of purified F5 scFv and diabody. F5 scFv and diabody were purified by IMAC and analyzed by gel filtration on a Sephadex 200 column. The mobility of the scFv was consistent with a molecular mass of 25 kDa, with no evidence of dimerization (the mobility of the diabody peak).

(Griffiths *et al.*, 1993). F5 bound recombinant ErbB2 with a $K_D = 1.6 \times 10^{-7}$ M as measured by surface plasmon resonance (SPR) in a BIAcore, and bound ErbB2 on SKBR3 cells with a $K_D = 1.36 \times 10^{-7}$ M. The K_D value of C1 was at least tenfold lower. Interestingly, these two scFv recognized the same epitope on ErbB2 as determined by competition ELISA (Figure 2(a)). This epitope was different than the epitope recognized by the human scFv phage antibody C6.5 and the murine monoclonal antibody 4D5 (Figure 2(a) and (b)). Since F5 and C1 recognized the same epitope, subsequent characterizations were performed using the higher affinity F5. F5 detected a band of the appropriate size for ErbB2 in a Western blot of the SKBR3 cell lysate (Figure 2(c)) and could immunoprecipitate ErbB2 from a SKBR3 cell lysate (Figure 2(d)).

Further characterization of non-ErbB2 binding antibodies

To characterize further the specificity of non-ErbB2 binding antibodies, phage were analyzed for binding to a panel of tumor and normal cell lines using flow cytometry. Phage were used for these studies rather than native soluble scFv because phage generate stronger signals due to signal amplification that results from the multiple copies of the major coat protein pVIII. To identify unique antibodies for flow cytometry studies, the scFv gene was PCR amplified from 18 SKBR3 positive and ErbB2 ECD negative clones and fingerprinted using the frequently cutting restriction enzyme *Bst*N1. Ten unique fingerprint patterns were identified representing ten unique antibodies. Phage were prepared from each different pattern and used to stain a panel of human cell lines (normal human fibroblasts, the breast tumor cell lines SKBR3 andMCF7, the ovarian tumor cell line SKOV3 and the prostate tumor cell line LNCaP). All ten phage antibodies stained SKBR3 cells better than fibroblasts as measured by flow cytometry (Table 3). Some phage antibodies stain all tumor cell lines (clones 3TE3, H7, 3TB5 and 2TF5) with a high intensity while others stain only subsets of cells (SKBR3 and SKOV3 cells: clones 3TH8 and 3TG5, SKBR3 and LNCaP cells: clone 3TF12, SKBR3 cells: clones 2TB4 and C2-1, or SKOV3 cells: clone 3GD8). We selected one of these phage antibodies that bound all tumor cells analyzed (H7) for further characterization.

H7 phage antibody binds the transferrin receptor

The H7 scFv gene was subcloned, expressed and purified by IMAC as described above. Like F5 and C1 scFv, gel filtration analysis indicated that H7 existed exclusively as monomeric scFv with no apparent spontaneous dimerization or aggregation. To identify the receptor bound by H7 scFv, initially we attempted to detect an immunoreactive band by Western blotting. However, no immunoreactive bands were apparent in a blot of SKBR3 cell lysate (Figure 2(c)). We therefore used the H7 scFv to immunoprecipitate membrane biotinylated SKBR3 cell extracts. For this experiment, H7 scFv was bound to Ni-NTA agarose, and biotinylated cell extracts were incubated with the loaded agarose beads. Bound immunocomplexes were eluted using immidazole, the eluted fractions run on an SDS-8% (w/v) PAGE, transfered onto nitrocellulose and blotted with a streptavidin-HRP conjugate. A major band running at 90 kDa (p90) was detected. The same procedure was used to quantitatively purifiy p90 from native cell lysates for N-terminal protein sequencing. The sequence corresponded to the N-terminal sequence of the transferrin receptor (TfR) (Schneider *et al.*, 1983). The identity of p90, was confirmed by analyzing immunoprecipitates of SKBR3 lysates obtained with H7 scFv using a monoclonal anti-TfR antibody (White *et al.*, 1992) (Figure 2(d)).

F5 and H7 phage antibodies and native scFv are rapidly internalized by SKBR3 cells

To determine whether F5 and H7 phage antibodies were endocytosed, we incubated phage with SKBR3 cells and identified internalized phage using an anti-pVIII antibody and confocal microscopy (Figure 3). Both F5 and H7 phage gave strong intracellular staining. In contrast, a control anti-botulinum phage antibody (Amersdorfer *et al.*, 1997) gave no intracellular staining and the anti-ErbB2 phage antibody C6.5 gave significantly less intracellular staining. This is consistent with pre-

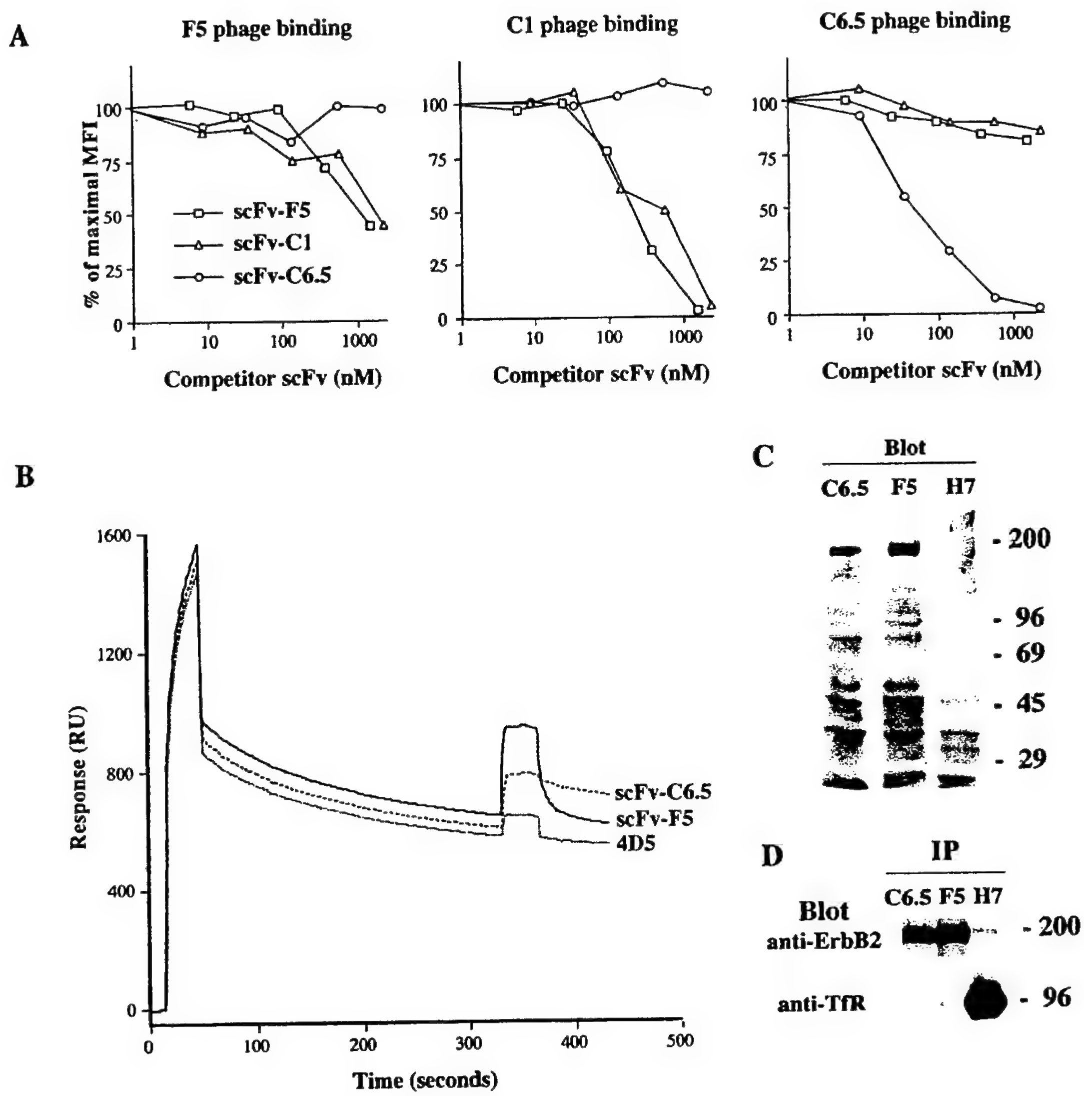

Figure 2. Characterization of anti-ErbB2 and anti-transferrin receptor scFv. (a) Epitope mapping of the F5 and C1 anti-ErbB2 scFv. The epitope recognized by the F5 and C1 scFv selected for internalization were compared to the ErbB2 epitope recognized by the scFv C6.5 selected on recombinant ErbB2 protein. For epitope mapping, the ability of purified C6.5, F5, and C1 scFv to block the binding of F5 phage (left panel), C1 phage (middle panel), and C6.5 phage (right panel) to ErbB2 expressing SKBR3 cells was determined by flow cytometry. F5 and C1 compete with each other for binding and recognize a distinct epitope from C6.5. (b) Epitope mapping of F5 and C6.5 scFv *versus* 4D5 IgG. The F5 and C6.5 epitopes were compared to the 4D5 epitope by BIAcore. 4D5 IgG was coupled to a sensor chip and ErbB2 was allowed to bind. The ability of C6.5 scFv, F5 scFv and 4D5 IgG to bind to ErbB2 was determined. Both C6.5 and F5 were able to bind, indicating a distinct epitope from 4D5. (c) Western blot of SKBR3 cell lysate using C6.5, F5, and H7 scFv. Both F5 and C6.5 recognize a band the appropriate size for ErbB2. No staining is seen with the H7 scFv. (d) Immunoprecipitation of ErbB2 and transferrin receptor from SKBR3 cell lysate using F5, C6.5 and H7 scFv. After immunoprecipitation with the appropriate scFv, lystates were run on SDS-PAGE, transferred to nitrocellulose and stained with either anti-ErbB2 or anti-transferrin receptor antibody. All scFv were able to immunoprecipitate their target antigen.

vious studies showing minimal endocytosis of C6.5 phage (Becerril *et al.*, 1999). Internalization was detected as soon as five minutes after application of F5-phage and 15 minutes after application of H7-phage (not shown). Purified and gel-filtered native scFv was also analyzed for internalization into SKBR3 cells by confocal microscopy, with endocytosed scFv detected with the monoclonal antibody 9E10 which recognizes the C-terminal myc-tag. As previously shown, both F5 and H7

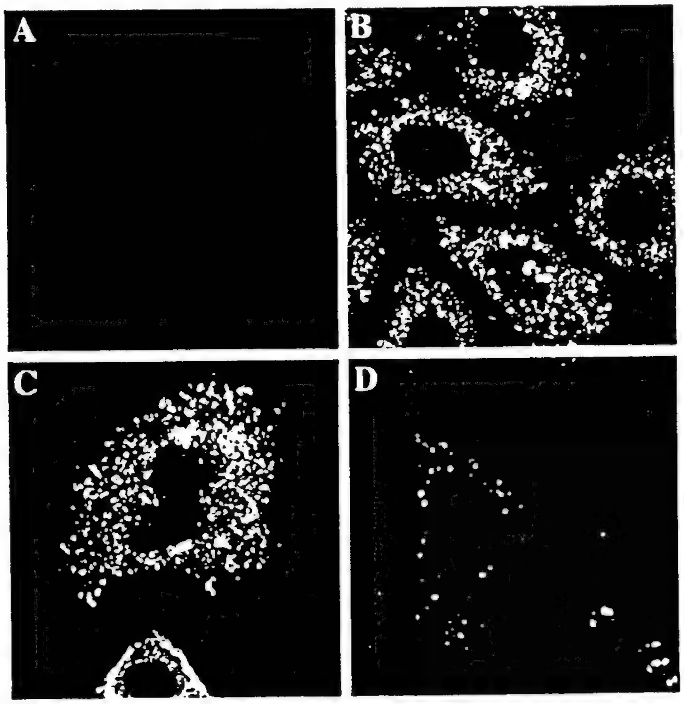

Figure 3. F5 anti-ErbB2 and H7 anti-transferrin phage are endocytosed by SKBR3 cells. Cells were incubated with either anti-ErbB2 phage antibodies F5 (b) and C6.5 (d), anti-transferrin receptor phage antibody H7 (c), or an irrelevant anti-botulinum phage antibody (a). Endocytosis was determined by staining with anti-M13 antibody and analyzing the results by confocal microscopy. Only F5 and H7 phage antibodies show significant intracellular staining.

scFv were monomeric in solution (Figure 1). Both F5 and H7 scFv gave strong intracellular staining, whereas no intracellular staining was seen using the control anti-botulinum scFv and minimal intracellular staining was observed with the anti-ErbB2 C6.5 scFv (Figure 4).

Growth inhibitory effects of F5 and H7 scFv on SKBR3 cells

Since the H7 and F5 antibodies bound to cell surface receptors in a manner that induced endocytosis, we evaluated whether there was any associ-

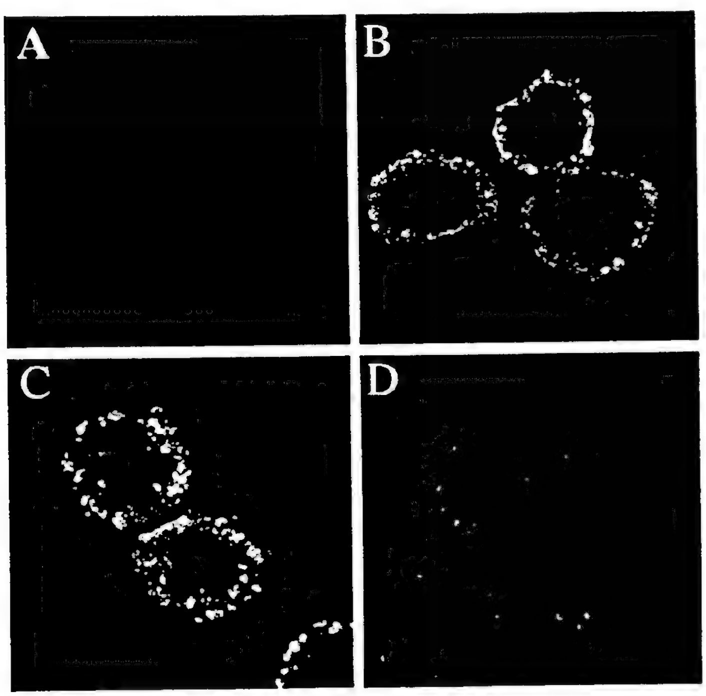

Figure 4. F5 anti-ErbB2 and H7 anti-transferrin receptor scFv are endocytosed by SKBR3 cells. Cells were incubated with either anti-ErbB2 scFv F5 (b) and C6.5 (d), anti-transferrin receptor scFv H7 (c), or an irrelevant anti-botulinum scFv (a). Endocytosis was determined by staining with an anti-myc tag antibody, which recognizes a C-terminal epitope tag on the scFv, and analyzing the results by confocal microscopy. Only F5 and H7 phage scFv show significant intracellular staining.

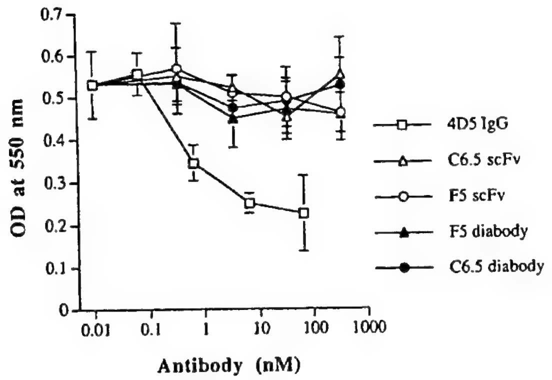

Figure 5. Effects of anti-ErbB2 antibodies F5, C6.5 and 4D5 on SKBR3 cell growth. The ability of F5 scFv, F5 diabody, C6.5 scFv, C6.5 diabody, and 4D5 IgG to inhibit the growth of SKBR3 cells was determined. Only 4D5 showed a dose-dependent growth inhibition.

ated biologic activity with respect to growth inhibition. As antibody-induced internalization can potentially increase the turnover of ErbB2 receptors, reduce the density of cell surface receptors and have an effect on cell growth (Sarup *et al.*, 1991; Tagliabue *et al.*, 1991), we tested the effects of F5 scFv on SKBR3 cell growth. F5 scFv had no effect on cell growth at concentrations up to 300 nM (10 μg/ml) while the control mAb 4D5 inhibited cell growth of 50% after five days of culture at a concentration of 5 nM as published (Sarup *et al.*, 1991) (Figure 5). Since no inhibitory effect had been observed with monovalent derivatives of growth inhibitory ErbB2 antibodies (Sarup *et al.*, 1991; Shawver *et al.*, 1994), we constructed a bivalent format of F5 scFv (diabody F5) by shortening the linker between the immunoglobulin heavy chain variable domain (V_H) and light chain variable domain (V_L) from 15 to five amino acid residues. This prevents intramolecular pairing of the V_H and V_L, resulting in intermolecular pairing and creation of an scFv dimer termed a diabody (Holliger *et al.*, 1993). The expected size of the F5 diabody was confirmed by gel filtration (Figure 1) and the functional affinity measured as 16 nM. The diabody F5 had no effect on SKBR3 growth (Figure 5). Similarly, neither scFv C6.5 or diabody C6.5 inhibited SKBR3 growth. In contrast the anti-ErbB2 antibody 4D5 showed dose-dependent growth inhibition as an IgG. While the results argue against a growth inhibitory effect for the F5 antibody, the distances between the antigen combining sites as well as binding site flexibility are different for diabodies and IgG. Whether F5 would cause growth inhibition as an IgG is unknown.

We also tested the ability of F5 scFv to induce downstream signaling upon ErbB2 binding. Starved CHO-ErbB2 cells were stimulated with monovalent (scFv) and bivalent (diabody) formats of F5. Both induced weak tyrosine phosphorylation of ErbB2 while the monoclonal antibody 4D5 induced strong phosphorylation (data not shown). The bivalent F5 diabody was also able weakly to activate the MAP kinase cascade as shown by SDS-PAGE band shift using an anti-Erk antibody (data not shown).

H7-scFv was also tested for SKBR3 growth inhibition in parallel with an irrelevant anti-botulinum scFv or with the 4D5 anti-ErbB2 mAb. We observed a strong inhibitory effect (50%) on cell growth using H7-scFv at a concentration of 300 nM (10 μg/ml). The extent of inhibition obtained was comparable to the maximal effect obtained using 4D5 and no inhibition was obtained with the irrelevant scFv (Figure 6(a)). Anti-TfR antibodies generated using hybridoma technology have also been associated with a growth inhibitory effect (Kovar *et al.*, 1995; Valentini *et al.*, 1994). To investigate the mechanism of the H7 scFv antagonist effect on cell growth, we studied the effect of holotransferrin (iron charged transferrin) on the binding of H7-phage antibodies to SKBR3 cells. Holotransferrin was able to inhibit H7 phage antibody binding to SKBR3 cells (IC_{50} 10 nM) (Figure 6(b)). Control experiments included inhibition of H7-phage binding with soluble scFv-H7

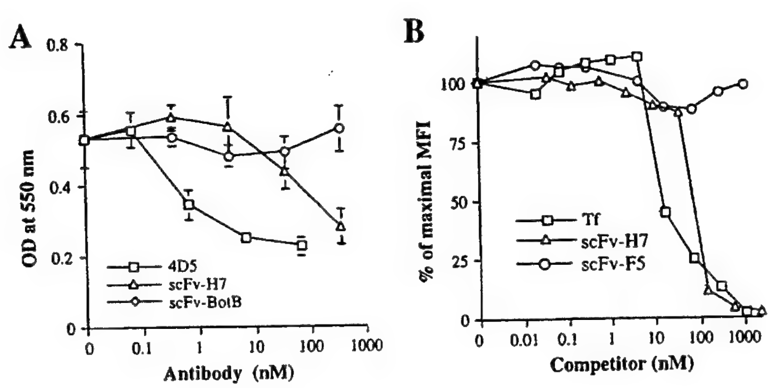

Figure 6. The anti-transferrin scFv H7 inhibits the growth of SKBR3 cells and is a mimic of the ligand holotransferrin. (a) Comparison of the growth inhibitory effect of anti-transferrin receptor H7 scFv and anti-ErbB2 IgG 4D5 on SKBR3 cells. Both antibodies exhibited dose-dependent growth inhibition. (b) Competition between H7 scFv and holotransferrin for binding to SKBR3 cells.

(IC_{50} 100 nM) and non-inhibition by irrelevant anti-ErbB2 F5 scFv. Holotransferrin also did not inhibit binding of SKBR3 cells by anti-ErbB2 F5-phage antibody. We conclude that the H7 scFv is an antagonist of transferrin binding to TfR. Transferrin, the physiological ligand of TfR, is a major carrier for iron and is rapidly internalized upon TfR binding. H7 scFv's inhibitory effect on SKBR3 growth may result from the combined effects of inhibition of holotransferrin endocytosis and of down regulation of TfR from the cell surface leading to intracellular iron depletion.

Comparison of internalization of F5 phage *versus* C6.5 phage

We have previously shown that C6.5 scFv displayed monovalently in a phagemid system was only minimally internalized, either as analyzed by confocal microscopy or by recovery of infectious phage from within the cytosol. In this system, enrichment ratios for C6.5 phagemids were only sevenfold above background, suggesting that successful selection from a library of monoclonal binders would be difficult. To understand better the successful selection of F5 and other monovalently displayed scFv from a phagemid library, we compared the internalization rate of F5 phagemid *versus* C6.5 phage with respect to recovery of infectious phage particles. After 120 minutes of incubation with 3.0×10^3 to 3.0×10^9 phage, significantly more F5 phagemid were recovered than C6.5 phagemid. In fact, F5 scFv displayed monovalently in a phagemid was taken up by ErbB2 expressing cells to a comparable extent as C6.5 scFv displayed multivalently in a phage vector (Figure 7).

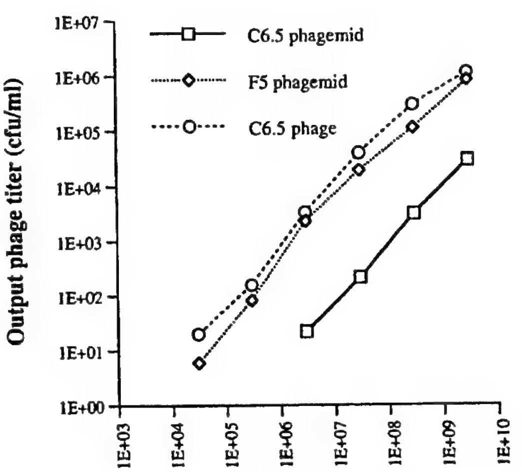

Figure 7. Titer of endocytosed phage as a function of applied phage titer. Varying concentrations of F5 phagemid, C6.5 phagemid, or C6.5 phage (input) were incubated with SKBR3 cells. Surface bound phage were removed with multiple low pH glycine washes and the titer on internalized phage (output) measured by infection of *E. coli*.

Discussion

Phage antibody libraries have become an important resource for the development of reagent, diagnostic, and therapeutic antibodies. Large non-immune libraries serve as a single pot resource for the rapid generation of human antibodies to a wide range of self and non-self antigens, including tumor growth factor receptors. Most of the antibodies isolated from combinatorial libraries expressed on phage have been selected using purified antigens or peptides immobilized on artificial surfaces. This approach may select antibodies that do not recognize the native protein in a physiologic context, especially with large molecular mass cell surface receptors. Attempts have been made to select antigen in native conformation using either cell lysates (Parren *et al.*, 1996; Sanna *et al.*, 1995; Sawyer *et al.*, 1997) fixed cells (Van Ewijk *et al.*, 1997) or living cells (Andersen *et al.*, 1996; Cai & Garen, 1995; de Kruif *et al.*, 1995; Marks *et al.*, 1993; Osbourn *et al.*, 1998; Siegel *et al.*, 1997). Such approaches, because of the heterogeneity of the starting material, require elaborate protocols including subtractive steps to avoid the selection of irrelevant antibodies. The few successful selections performed on heterogenous material were generally done using small libraries from immunized sources. There are only three reports of successful selection on cells using large non-immune libraries (de Kruif *et al.*, 1995; Marks *et al.*, 1993; Vaughan *et al.*, 1996). The use of immunized libraries limits the spectrum of antigen specificities that can potentially be obtained from the same library and typically yield murine antibodies.

The step limiting the selection of binders from large naïve libraries by cell panning seems to be the relatively high background binding of non-specific phage and relatively low binding of specific phage (Becerril *et al.*, 1999; Pereira *et al.*, 1997; Watters *et al.*, 1997). The low binding of specific phage is partially related to the low concentration of a given binding phage in the polyclonal preparation (approximately 1.6×10^{-17} M for a single member of a 10^9 library in a phage preparation of 1.0×10^{13} particles/ml). The low concentration simultaneously limits the efficiency of both subtraction of common binders and enrichment of specific binders. To overcome this limitation, we sought to take advantage of normal cell surface receptor biology. Many receptors undergo endocytosis upon ligand binding. Antibodies can mimic this process, causing receptor aggregation and endocytosis of the antibody upon binding. We hypothesized that enrichment ratios of specific binders could be significantly increased by recovering endocytosed phage antibodies from the cytosol after stringent removal of non-specific phage from

the cell surface. Using a model system employing an anti-ErbB2 phage antibody, we found that enrichment of specific *versus* non-specific phage ranged from 3.5 to 146-fold for endocytosed phage compared to 2.7 to 20-fold for surface-bound phage (Becerril *et al.*, 1999). However, the highest values were found only for dimeric antibody species, either dimeric diabodies displayed monovalently in a phagemid vector or scFv displayed multivalently in a phage vector. This is not surprising, since the literature indicates that with rare exceptions, antibodies must be bivalent IgG to induce receptor cross-linking and endocytosis. All large non-immune libraries display monovalent antibody fragments (either scFv or Fab) as single copies using a phagemid vector. Thus, successful selection of internalizing antibodies from such libraries would either require that: (1) the scFv formed spontaneous diabody dimers, as has been reported for some scFv; (2) the monovalent scFv mimicked the natural receptor ligand leading to receptor aggregation and endocytosis; or (3) increased phage display levels of some scFv resulted in greater than one scFv per phage.

Here, we report the successful application of this methodology to select internalizing antibody fragments from scFv libraries displayed monovalently on phage. A large panel of scFv were selected by panning on the tumor cell line SKBR3 which does not recognize normal human fibroblasts. The relatively small number of scFv analyzed have differing patterns of reactivity for other tumor cell lines. Based on the diversity of binders observed in the small sample analyzed (ten different antibodies out of 18 analyzed), hundreds to thousands of different binders with different specificities are likely to be present. To understand better the properties of the selected antibodies, we studied three in detail, two anti-ErbB2 and one that was determined to bind the transferrin receptor. All three were efficiently endocytosed into the target cell line, both as phage and as native monomeric scFv antibody fragments. Somewhat to our surprise, the three scFv did not spontaneously dimerize or aggregate as an explanation for their efficient endocytosis Rather, the data suggest that some scFv, such as the anti-transferrin receptor antibody, act as ligand mimetics resulting in conformational receptor changes which trigger endocytosis. This may also be the case with the anti-ErbB2 scFv; however, this cannot be studied, since the natural ligand for ErbB2 homodimerization is unknown. Since the anti-ErbB2 scFv recognize ErbB2 in a Western blot, it is unlikely that they are endocytosed by binding an epitope present only on dimerized ErbB2.

When compared to the model C6.5 anti-ErbB2 scFv, the internalizing anti-ErbB2 F5 scFv was endocytosed as efficiently when displayed monomerically in a phagemid system as C6.5 displayed multivalently on phage. This result explains how we were able successfully to select internalizing antibodies from an scFv phagemid library and reconciles our results with observations from the model system. Our results indicate that selection of antibody fragment libraries displayed on phagemid yields antibodies which are endocytosed as monomers. This is likely to be only a small subset of antibodies capable of triggering receptor-mediated endocytosis, limited to those antibodies capable of mimicking natural ligand binding or otherwise inducing conformational receptor changes leading to receptor aggregation. Most antibodies require a multivalent format to induce receptor cross-linking and endocytosis. Thus, construction of diabody libraries in a phagemid vector or scFv or Fab libraries in a phage vector (Griffiths *et al.*, 1994) should open up this selection approach to more epitopes on more target antigens. Our model system results indicate that the most efficient selection format would be display on phage, an approach which is presently under investigation.

The major advantage of selecting for internalizing antibodies is that one selects for antibodies that trigger a biologic function, not just antibodies that bind. In this case the biologic function is receptor-mediated endocytosis. Such antibodies are likely to have significant therapeutic utility. Use of receptor-mediated endocytosis as a drug delivery route allows delivery of the therapeutic agent specifically into target cells that overexpress the receptor, thereby increasing efficacy while reducing systemic toxicity. In addition, many "drugs" require delivery into the cell for activity (for example, genes and toxins). In some instances, internalization can also be used as a surrogate marker for desirable biological effects of the antibody, for example apoptosis, growth inhibition or growth stimulation. Indeed, we observed a significant growth inhibitory effect of the anti-transferrin scFv on cancer cells. Thus, antibodies selected using this approach may have a direct therapeutic effect, as well as the ability to deliver drugs into the cytosol. Since many antibodies generated by conventional means are not endocytosed, this selection strategy provides a more efficient route to generating internalizing antibodies compared to selecting on protein antigens and screening antibodies for endocytosis. For example, screening the same non-immune library on recombinant ErbB2 extracellular domain did not yield either the F5 or C1 internalizing scFv, perhaps because their K_D values were significantly higher than other anti-ErbB2 scFv isolated.

As an indicator of potential therapeutic utility of antibodies selected for internalization, we have conjugated the F5 anti-ErbB2 scFv to the surface of commercial liposomal doxorubicin converting it into fully functional doxorubicin-loaded anti-ErbB2 immunoliposomes (Nielsen *et al.*, unpublished results). The resulting immunoliposomes have superior efficacy in ErbB2 overexpressing mouse xenograft models compared to untargeted liposomal doxorubicin. Based on preclinical data, expression of the F5 scFv has been scaled up for toxicology studies, cGMP manufacture, and an anticipated phase 1 clinical trial in breast cancer commencing in 2001 (Glaser, 1998).

In summary, we have developed a method for selecting internalizing antibody fragments from phage antibody libraries. The approach can be used to generate internalizing antibodies to known receptors and to identify novel cell surface receptors. The antibodies generated can be used to target therapeutic molecules to the cytosol and in some instances will exert a direct cellular biologic effect *via* their ability to modulate receptor function.

Materials and Methods

Cell culture

Normal human fibroblasts and MCF7 cells were grown in DMEM, 10% (v/v) fetal bovine serum (FBS) (Hyclone), normal human breast cell line Hs 518Bst (ATCC) in DMEM, 10% fetal calf serum (FCS) complemented with 10μg/ml bovine insulin and 30 ng/ml epidermal growth factor (EGF), SKBR3 in RPMI, 10% FBS, CHO in F12, 10% FBS and CHO-EGFR (Morrison *et al.*, 1993) and CHO-ErbB2 (a gift from Keith Marshall) in F12, 10% complemented with 0.5 mg/ml G418.

Selection of internalizing phage antibodies

A total of five million freshly trypsinized normal human fibroblasts and 10^{12} cfu of the phage library (Sheets *et al.*, 1998) were diluted in 10 ml of ice-cold RPMI, 10% FCS and added to sub-confluent SKBR3 cells grown in a 10 cm diameter plate. After 1.5 hours of incubation at 4 °C on a rocker, the cells were washed six times with PBS, covered with prewarmed culture medium and returned to 37 °C. After 15 minutes, the cell surface was stripped by three incubations of ten minutes with 4 ml of glycine buffer (500 mM NaCl, 0.1 M glycine (pH 2.5)). The cells were then trypsinized washed with 50 ml of PBS again, lysed with 1 ml of 100 mM TEA for four minutes at 4 °C and neutralized with 0.5 ml of 0.5 M Tris (pH 7.4). The phage content of the TEA lysate and the first two glycine washes (neutralized with 1 ml of 0.5 M Tris (pH 7.4)) was titered by infection of *Escherichia coli* TG1 to monitor the selection. Internalized phage (TEA lysate) were amplified for another round of selection. Three rounds of selection were performed.

Initial characterization of binders by ELISA

After two and three rounds of selection, soluble scFv was expressed (De Bellis & Schwartz, 1990) from single colonies grown in 96-well microtiter plates as described (Marks *et al.*, 1991). Crude culture supernatant were tested in ELISA for ErbB2 binding as described (Schier *et al.*, 1996a). In parallel, the bacterial supernatant was tested by cell ELISA on SKBR3 cells and on fibroblasts. Cells were distributed in conical 96-well plates (500,000 cells per well) and then centrifuged at 300 *g* for three minutes. The cell pellet was resuspended in 100 μl of bacterial supernatant diluted twofold with PBS, 4% (v/v) skimmed milk and incubated for one hour at 4 °C on a rocker. After two washes with cold PBS (done by resuspending the cell pellet in 180 μl of PBS and a three minute centrifugation at 300 *g*), the bound scFv were detected *via* their C-terminal myc-tag (Munro & Pelham, 1986) using the monoclonal antibody 9E10 and peroxidase conjugated anti-mouse Fc (Sigma). The diversity of ELISA positive clones was determined by PCR amplifying and DNA fingerprinting the scFv gene with *Bst*N1 as described (Marks *et al.*, 1991). Unique scFv fingerprint patterns were sequenced using a Sequitherm sequencing kit (Epicentre).

ScFv expression and purification

To facilitate purification of soluble scFv, the scFv genes were subcloned into the expression vector pUC119mycHis (Schier *et al.*, 1995) resulting in the addition of a hexahistidine tag at the C-terminal end of the scFv. The scFv were purified from periplasmic fractions of *E. coli* TG1 by IMAC (Hochuli *et al.*, 1988), using a Ni-NTA column (Qiagen), and gel filtration, as published (Schier *et al.*, 1996b) except that the running buffer after gel filtration was PBS instead of hepes-buffered saline (HBS) for cell culture applications. Alternatively, the scFv genes were PCR amplified using the primer LMB3 (Marks *et al.*, 1991) and fd-FLAG primers before subcloning into pUC119mycHis, resulting in the addition of the flag tag at the N terminus of the scFv.

Immunofluorescence

Cells were grown on coverslips to 50% of confluency in six well-plates and incubated with phage particles or purified scFv for two hours at 37 °C. The coverslips were washed six times with PBS, three times for ten minutes with glycine buffer (50 mM glycine (pH 2.8), 500 mM NaCl), neutralized with PBS and fixed with PBS containing 4% (w/v) paraformaldehyde for five minutes at RT. Cells were permeabilized with cold acetone for 30 seconds. and saturated with PBS, 1% BSA. Antibodies were diluted with saturation solution. Intracellular phages were detected with biotinylated anti-M13 polyclonal antibody directed against the pVIII major phage coat protein (5 Prime, 3 Prime Inc.) and streptavidin-Texas Red conjugate (Amersham) both diluted 300 times. Intracellular scFv were detected using the 9E10 mAb (1 μg/ml) (Santa Cruz), an anti-mouse biotinylated antibody (Amersham; diluted 200 times) and streptavidin-Texas Red. Coverslips were inverted on a slide on mounting medium and optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Immunofluorescent microscopy was performed with a Zeiss Axioskop UV fluorescent microscope.

Analysis of phage binding by flow cytometry

Experiments were performed at 4 °C. Aliquots of 100,000 cells resuspended in FACS buffer (PBS, 1% FBS) were distributed in conical 96-well microtiter plates and incubated with 100 μl of phage antibodies (typically titering about 5.0×10^{12} cfu/ml) diluted in PBS, 4% milk for one hour at 4 °C. After two PBS washes, bound phage were detected by resuspending the cell pellet in 100 ml of biotinylated anti-M13 sheep antibody diluted 300 times in FACS buffer (30 minutes). Cells were washed again and incubated with streptavidin-phycoerithrine conjugated (PE) (Jackson) for 15 minutes and analyzed using a FacScan (Becton Dickinson). For competition experiments, SKBR3 cells were preincubated with various concentrations of soluble scFv or holotransferrin (Sigma) for one hour at 4 °C. Phage antibodies were added (titer between 10^9 and 5.0×10^9 cfu per

well), incubated one hour at 4 °C and bound phage detected as described above.

Affinity measurement and epitope mapping with the BIAcore

On and off-rates were determined using SPR in a BIAcore1000. Approximately 800 RU of ErbB2 ECD were coupled to a CM5 sensor chip as described (Schier, 1995). Association and dissociation rates were measured under continuous flow of HBS at 15 µl/minute using concentrations ranging from 100 nM to 1200 nM and calculated using the BIAanalysis software. For epitope mapping, mAb 4D5 was diluted to 10 µg/ml in 10 mM sodium acetate (pH 4.5), for direct immobilization of 3000 RU to the chip surface.

Affinity measurement on cells

SKOV3 cells were grown to 80-90% confluence in RPMI supplemented with 10% FCS. Cells were harvested by trypsinization. ScFv were incubated with 1×10^5 cells for two hours at varying concentrations. Cell binding was at room temperature in PBS containing 1% (w/v) BSA and 0.1% (w/v) sodium azide in a total volume of 200 µl. After two washes in PBS/BSA, bound scFv was detected with saturating amounts of FITC-labeled anti-FLAG clone M1 (10 µg/ml). After 30 minutes of incubation cells were washed twice and resuspended in PBS containing 1% paraformaldehyde. Fluorescence was measured in a FACSort™ and median fluorescence calculated using the Cellquest™ software and K_D calculated (Benedict *et al.*, 1997).

Cell growth inhibition assay

A total of 100 µl of 10^5 cells/ml were plated in 96-well plates. Four hours later, 100 µl of antibody solutions diluted in culture medium were added and cells incubated for three to five days. The number of living cells was estimated using the CellTiter 96 AQueous cell growth assay kit (Promega).

Western blot and immunoprecipitation using scFv antibodies

SKBR3 cell extracts were prepared using 0.5 ml of lysis buffer (0.4% (v/v) NP40, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM DTT, 1 mM PMSF, aprotinin, leupeptin) per confluent 10 cm plate. Cell lysates were run on a SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with scFv (10 µg/ml in PBS, 0.05%, Tween 1% BSA) overnight at 4 °C. Blots were washed and scFv detected using 9E10 anti-myc tag antibody (0.1 µg/ml) and HRP conjugated anti-mouse Ig (Amersham). For immunoprecipitation, a dialyzed periplasmic fraction containing the scFv from a 500 ml culture of *E. coli* TG1 was loaded onto 500 µl of a Ni-NTA agarose column. The beads were washed once with PBS, 35 mM imidazole. Then 100 µl of the scFv-loaded Ni-NTA agarose (50% slurry) was used to immunoprecipitate 0.5 ml of SKBR3 cell extract. Immunoprecipitates were analyzed by Western blotting using scFv-F5 or scFv-H7, anti-ErbB2 (Santa Cruz) or anti-human transferrin receptor (TfR) H68.4 mAb (White *et al.*, 1992) (a gift from Keith Mostov, UCSF). Alternatively, the cell surface was biotinylated prior to cell lysis and immunoprecipitation. Cells (from a 10 cm diameter confluent plate) were washed twice with cold PBS and incubated with 3 ml of a 0.1 mg/ml solution of Sulfo-NHS-LC-biotin (Pierce) freshly dissolved in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ at 4 °C for 20 minutes. The reaction was quenched by two washes with cold PBS, 50 mM glycine. After a final wash with PBS, cells were lysed with 0.5 ml of lysis buffer. Immunoprecipitation was performed as described above and analyzed by Western blot using HRP-conjugated streptavidin.

Purification of antigen using scFv antibodies

A total of 200 µl of a scFv-H7-Ni-NTA agarose column were loaded twice with a SKBR3 cell lysate corresponding to 3.0×10^6 cells. The column was washed with PBS, 35 mM immidazole, and resuspended directly in 100 µl of Laemli loading buffer 4 ×. The immunoprecipitate was run on a 6% gel, transferred onto PVDF membrane and stained with Ponceau S. The N-terminal protein sequence was determined by Edman sequencing.

Signaling studies

Confluent CHO-ErbB2 cells grown in 6 cm diameter plates were serum starved overnight and stimulated with antibodies for five minutes or one hour and lyzed in 300 µl of lysis buffer complemented with sodium orthovanadate. The ErbB2 phosphorylation level was analyzed by Western blot using the anti-phosphotyrosine mAb 4G10 (UBI) and HRP-conjugated anti-mouse IgG (Amersham). ErbB2 levels were checked with the anti-ErbB2 C-18 rabbit polyclonal antibodies (Santa Cruz). MAPkinases Erk1 and Erk2 were detected using the anti-Erk1 K-23 antibody (Santa Cruz) that cross-reacts with Erk2.

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Therapeutic efficacy of anti-ErbB2 immunoliposomes targeted by a phage antibody selected for cellular endocytosis

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Abstract

Many targeted cancer therapies require endocytosis of the targeting molecule and delivery of the therapeutic agent to the interior of the tumor cell. To generate single chain Fv (scFv) antibodies capable of triggering receptor mediated endocytosis, we previously developed a method to directly select phage antibodies for internalization by recovering infectious phage from the cytoplasm of the target cell. Using this methodology, we reported the selection of a panel of scFv that were internalized into breast cancer cells from a non-immune phage library. For this work, an immunotherapeutic was generated from one of these scFv (F5) which bound to ErbB2 (HER2/*neu*). The F5 scFv was re-engineered with a C-terminal cysteine, expressed at high levels in *E. coli.*, and coupled to sterically stabilized liposomes. F5 anti-ErbB2 immunoliposomes were immunoreactive as determined by surface plasmon resonance and were avidly internalized by ErbB2 expressing tumor cell lines in proportion to the levels of ErbB2 expression. F5-scFv targeted liposomes containing doxorubicin had anti-tumor activity and produced significant reduction in tumor size in xenografted mice compared to non-targeted liposomes containing doxorubicin. This strategy should be applicable to generate immunotherapeutics for other malignancies by selecting phage antibodies for internalization into other tumor types and using the scFv to target liposomes or other nanoparticles.

Introduction

Many targeted therapeutic approaches to cancer require endocytosis of the targeting molecule and delivery of the therapeutic agent to the interior of the tumor cell. Such drug targeting strategies rely on the use of ligands capable of binding to tumor cell surface receptors in a manner that triggers receptor endocytosis. Antibodies have been successfully used as surrogate receptor ligands for intracellular targeting of toxins [1], liposomes [2], drugs and DNA [3]. Currently, antibodies that trigger internalization are identified by screening antibodies derived by either hybridoma or phage antibody technology [4]. This usually involves generating and examining antibodies recognizing specific targets. While such approaches may take the biology of the target receptor into account, they do not necessarily select for the requisite biology of the antibody, triggering receptor endocytosis. This limits the availability of useful targeting antibodies, since many surface receptors do not undergo endocytosis and for those that do, the efficiency with which an antibody mediates internalization can vary significantly depending on the epitope recognized [5, 6].

We recently developed a strategy to directly select internalizing antibodies from phage libraries by recovering infectious phage from within a target cell line [7]. The technique was applied to select a panel of antibodies from a naïve phage library [8] that were specifically endocytosed into the breast tumor cell line SK-BR-3 [9]. Upon further characterization, two of the antibodies (F5 and C1) were identified as binding ErbB2, a growth factor receptor overexpressed in 20-30% of human breast carcinomas as well as other adenocarcinomas. In this work we demonstrate how such scFv antibodies can be used to construct a targeted drug with potent anti-tumor activity. Specifically, we

engineered the anti-ErbB2 F5 scFv for conjugation to amphipathic PEG for construction of immunoliposomes by membrane capture into preformed stealth liposomes, yielding anti-ErbB2 immunoliposomes. Such F5 immunoliposomes are efficiently endocytosed by ErbB2 expressing tumor cell lines and have potent anti-tumor activity in xenografted mice, which significantly exceeds that of untargeted doxorubicin containing liposomes. This strategy of selecting internalizing antibodies from phage libraries and construction of immunoliposomes provides a generic, rapid and facile route for making targeted therapeutics for many types of tumors.

Methods

Construction of expression vector pELK

A fragment containing the pelB leader and cloning sites from vector pUC119mycHis [10] was amplified using standard PCR conditions. Primers were designed to append a 5' *Xba*I site and a 3' *Sal*I site for cloning into pET9a (Promega). The alkaline phosphatase promoter (PhoA) from *E. coli* was then cloned as an *Eco*RI/*Xba*I fragment yielding the pELK expression vector.

F5 scFv expression

A C-terminal cysteine followed by a stop codon was added to the F5 scFv by PCR using primers G4C-NotI (5'-GCT CTA GAT CAG CAG CCT CCA CCG CCA CCT AGG ACG GTC AGC TTG GTC CC-3') and LMB3 [11]. The PCR product was cloned into the *Nco*I/*Not*I sites of vector pELK. ScFv was expressed from *E. coli* strain RV308 (ATCC) by fermentation, essentially as described in ref. [12]. Soluble protein obtained as a periplasmic extract [12] was purified on recombinant protein A agarose (Pharmacia).

Conjugation of F5 scFv to maleimide-PEG-DSPE

F5 scFv was reduced on a thiol-reducing column (ReduceImm, Pierce) according to the manufacturers' instructions, except guanidine was omitted from the running buffer and reduction time was 30 min at room temperature. Alternatively, F5 scFv was incubated in 144 mM NaCl, 20 mM MES, pH 6.0 in the presence of 10 mM cysteamine hydrochloride and 5 mM EDTA at 32°C for 1 hour, and isolated by passage through a desalting column (PD-10, Pharmacia). The number of free thiol groups per molecule was

determined with Ellman's reagent as described by the manufacturer (Pierce). The reduced scFv was incubated overnight at 4°C with maleimido-PEG_{2000}-distearoyl-phosphatidylethanolamine (Avanti Polar Lipids) in 144 mM NaCl aqueous buffer at pH 6.5-7.5 at a protein/linker molar ratio of 1:4. The amounts of conjugated and free protein were determined by SDS-PAGE with Coomassie staining and band densitometry. For *in vitro* studies, and to afford quantitation of liposome-conjugated scFv, 1 mg of scFv (prior to conjugation) was labeled with 0.3 μCi of ^{125}I using IodoBeads (Pierce) as described by the manufacturer.

^{125}I-labelling and internalization study

Purified F5 scFv was labeled with ^{125}I as above and incubated (10 μg/mL) with live SK-BR-3 cells in 12-well plates (150,000 cells/well) for 15, 30, 60, or 90 minutes at 37°C. After washing four times with Hanks' balanced salt solution without Phenol Red (HBSS; Gibco), cell surface bound antibody was removed by two 10 min washes with 1 mL glycine buffer (glycine 50 mM, pH 2.8, adjusted to the osmolarity of 290 mmol/kg with NaCl). Cells were lysed in 0.1% Triton-100 and internalized and cell surface associated radioactivity measured in a Packard gamma counter. Kinetics of endocytosis was fitted using Berkeley Madonna™ differential equations solver (available at www.berkeleymadonna.com).

Liposome preparation

For *in vitro* assays, liposomes were prepared from 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), cholesterol, and methoxy-poly(ethylene glycol (M.w.

2,000)-distearoylphosphatidylethanolamine (PEG_{2000}-DSPE) (3:2:0.3 molar ratio) (all from Avanti Polar Lipids) by lipid film hydration in a solution containing membrane-impervious fluorescent marker, followed by extrusion through track-etched polycarbonate membranes with pore size 100 nm [2]. For internalization studies, the solution contained pH-sensitive marker trisodium 8-hydroxypyrene-1,3,5-trisulfonate (HPTS); 35 mM, pH 7.0 [2, 13]. For fluorescence microscopy, the marker was 20 mM BODIPY disulfonate (both probes from Molecular Probes). Both dye solutions were adjusted to the osmolarity of 290 mmol/kg with NaCl. For *in vivo* therapeutic efficacy studies, Doxil (Alza Corp.) obtained commercially was used for immunoliposome formation. Doxil contains unilamellar liposomes (70-90 nm) composed of hydrogenated soy phosphatidylcholine, cholesterol, and PEG^{2000}-DSPE (3:1:0.3 by mass) and doxorubicin (0.15 mg/μmol of phospholipid) encapsulated into the liposomes by an ammonium sulfate gradient method [14, 15].

Immunoliposomes were formed by overnight incubation of the PEG-DSPE-conjugated F5 scFv with preformed liposomes at 37°C, followed by the removal of non-incorporated F5 conjugate, non-conjugated scFv, and any extraliposomal low-molecular weight molecules by gel chromatography using a Sepharose 4B column (Pharmacia). The liposomes were excluded from the gel and recovered in the void volume fraction. Separation of micelles from liposomes was confirmed as follows: 0.5 mL of F5-PEG-DSPE (1.6 mg/mL protein) or 0.5 mL of doxorubicin liposome (Doxil) diluted to 0.2 mg/mL of doxorubicin with HEPES-buffered saline (5 mM HEPES, 144 mm NaCl, pH 7.4) were applied onto a column (diameter 1.5 cm, bed volume 13 mL) with Sepharose 4B (Pharmacia Amersham) and eluted with HEPES-buffered saline. Fractions (0.5.ml)

were collected and spectrophotometrically analyzed for F5 protein (absorbance at 280 nm) or doxorubicin (absorbance at 485 nm). The amounts of F5 or doxorubicin per fractions were expressed as % of total applied. Total recovery of both F5-PEG-DSPE and doxorubicin liposomes in the eluted fractions was >97%. To afford quantification of liposome-linked F5, the protein was labeled with ^{125}I, purified by gel chromatography, reduced, and finally conjugated to maleimido-PEG-DSPE. The resulting ^{125}I-F5-PEG-DSPE conjugate was mixed with the unlabeled conjugate at a ratio of 1:9 and subsequently incubated with POPC/Cholesterol/PEG-DSPE liposomes at various protein-to-liposome ratios (2-100 scFv/liposome). Liposome concentration was determined by phosphate analysis adapted from Morrison [16]. Doxorubicin in the liposome and immunoliposome preparations was quantified by spectrophotometry at 485 nm after solubilization of liposomes in 70% aqueous isopropanol-0.1 N HCl

Immunoliposome analysis by surface plasmon resonance

Binding activity of immunoliposomes was determined by surface plasmon resonance in a BIAcore1000 instrument (BIAcore Inc.). Approximately 3000 RU of ErbB2 ECD were coupled to a CM5 sensor chip (BIAcore Inc.) as described [10] and binding rates were measured under continuous flow of 15 μL/min. Immunoliposomes were injected at a concentration of 50 μM phospholipid. To determine the kinetics of immunoliposome binding to ErbB2, serial dilutions of recombinant ECD were incubated with 50 μM immunoliposomes for one hour prior to binding analysis. Binding activity was determined from a linear standard curve of binding slope versus concentration.

Flow cytometry analysis

Human cancer cells SK-BR-3, SK-OV-3, BT474, MCF7, MDA-MB-453, MDA-MB-231, MDA-MB-468 (ATCC) were grown to 80-90% confluence in the media type recommended by ATCC and supplemented with 10% fetal calf serum (FCS). The ErbB2 transfected cell line MCF7/HER2 was grown as previously described [17]. F5 scFv (20 μg/mL), containing a version of the FLAG epitope tag with improved affinity [18, 19], was used to detect the levels of ErbB2 expression by flow cytometry as previously described [18].

Spectrofluorometric measurement of immunoliposome uptake by cells.

Tumor cells were grown in 96-well plates to subconfluency. To determine cellular uptake or kinetics of internalization, immunoliposomes containing HPTS were incubated at 200 μM total phospholipid in a 37°C incubator in complete cell culture media for the indicated times. After washing four times with Hanks' balanced salt solution without Phenol Red, fluorescence was read at 512 nm following excitation at 414 nm and 454 nm, in a Gemini microfluorometer (Molecular Devices). Total cellular uptake of liposomes was calculated from the fluorescence at isosbestic point (414 nm), and the amount of endocytosed liposomes was determined from the fluorescences at 454 nm and 414 nm excitation as described [13, 20].

Fluorescence microscopy

SK-BR-3 cells were grown in 12-well plates to subconfluency. Immunoliposomes containing approximately 60 scFv per liposome and loaded with BODIPY disulfonate (final concentration 100 μM phospholipid) were incubated with SK-BR-3 cells for 2 hours at 37°C in complete media along with 5 μg/mL of tetramethylrhodamine-labeled transferrin (Molecular Probes). The cells were then washed six times in HBSS and live cells were examined through a Nikon Eclipse 300 inverted fluorescence microscope.

In vivo antitumor efficacy studies

Antitumor efficacy studies were preformed as previously described [25]. Briefly, tumor xenografts were raised subcutaneously (s.c.) in 5-week old female homozygous nude mice (NCR nu/nu) using a subline of BT474 human breast adenocarcinoma cells (ATCC HTB-20) which contain about 10^6 ErbB2 receptors/cell [21]. The subline (BT474M1) with increased tumorigenicity was derived from BT-474 xenografts selected for maximum growth rate [25]. The animals were inoculated s.c. at the base of the right scapula with $2x10^7$ BT474M1 cells in 0.1 mL of the growth medium. Two days prior to inoculation, a 60-day sustained release 17β-estradiol pellet (0.72 mg; Innovative Research of America) was implanted s.c. near the base of the tail [22]. When tumor xenografts had become fully established (300-400 mm^3), the mice were randomly assigned to treatment groups of 15 animals per group. F5-immunoliposomal doxorubicin or liposomal doxorubicin (Doxil) were administered intravenously at 5 mg doxorubicin /kg once weekly for three consecutive weeks (total doxorubicin dose of 15 mg/kg). Control group received i.v. injections of the excipient (HEPES-buffered saline, HBS) at

the same schedule as liposomes and immunoliposomes. Tumors were measured twice weekly using a caliper, and tumor volumes were calculated by multiplying length X width X thickness of the tumor and expressed as the mean for each group (mm^3 +/- SE). Statistical significance of the differences between tumor sizes among the groups was determined by Student's t-test.

Results

Isolation of tumor specific scFv antibodies by selection for internalization

A naïve scFv phage antibody library containing 7×10^9 members [8] was selected for endocytosis into SK-BR-3 tumor cells [7, 9]. In this selection strategy, the phage antibody library was allowed to bind to the cell surface of live SK-BR-3 cells at 4°C, unbound phage was removed by washing and cells were subsequently incubated at 37°C for 15 minutes to allow internalization of the phage. Phage remaining on the cell surface were removed with acid washes and internalized phage recovered by infection of the cell lysate into *E coli*. This strategy allowed the isolation of rapidly internalizing phage antibodies [7, 9]. After three rounds of selection, we identified more than 40 distinct scFv that internalized into SK-BR-3 cells. One of these antibodies, F5, was shown to recognize the extracellular domain (ECD) of ErbB2 with a K_D of 160 nM and was efficiently endocytosed into SK-BR-3 cells as phage and as purified monomeric F5 scFv [9].

Kinetics of internalization into tumor cells

The kinetics of internalization into live SK-BR-3 tumor cells overexpressing ErbB2 was investigated using F5 scFv labeled with ^{125}I. Following incubation from 0 - 90 minutes, cell surface bound antibody fragments were stripped with low pH glycine buffer and the fraction of internalized and cell surface bound antibody determined by measuring radioactivity. A plot of time versus the amount of internalized antibody fragment allowed determination of the rate of internalization (results not shown). The internalization rate was 0.0056 min^{-1} for the F5 scFv.

Re-engineering and expression of the scFv for construction of immunoliposomes

To provide a single thiol group as a convenient and unique site for conjugation to a drug delivery carrier, the F5 scFv was re-engineered by PCR to contain the C-terminal sequence Gly-Gly-Gly-Gly-Cys followed by a stop codon, and was cloned into the expression vector pELK. This vector consists of the pET9 backbone with an alkaline phosphatase promoter cloned in place of the pET9 promoter. The scFv containing the C-terminal cysteine expressed at high levels (>20 mg/L) by fermentation in *E. coli*. The F5 scFv sequence belongs to the V_H3-gene family of which approximately 50% bind Protein A [23]. This was the case for the F5 scFv, allowing for a one-step affinity purification on immobilized protein A, following which the recombinant protein was more than 90% pure as judged by SDS-PAGE (Figure 1).

Conjugation of the F5 scFv to a lipid linker and formulation into liposomes

Immunoliposomes are usually made by direct conjugation of the antibody to liposomes formulated with activated lipid anchors for coupling of antibody to the liposome surface [24]. However, construction of immunoliposomes by membrane capture of amphipathic antibody-PEG conjugates into preformed liposomes offers a new and efficient method of immunoliposome construction [25, 26].

Initial preparations of the F5 scFv contained approximately 0.1 free thiol groups per molecule and existed as a mixture of monomers and covalent dimers (Figure 1, lane 1). Therefore this protein was reduced to make the thiol group of the C-terminal cysteine

available for conjugation. Following elution from a thiol-reducing column, or by treatment with cysteamine hydrochloride at pH 6.0, a majority of the dimer was reduced to the monomeric form (Figure 1, lane 2), resulting in 0.7-1.2 free thiol groups per molecule for conjugation to an amphipathic linker, N-(ω-(N-maleimido)-poly(ethylene glycol)$_{2000}$-α-oxycarbonyl-distearoyl phosphatidylethanolamine (PEG$_{2000}$-DSPE). Coupling of the reduced protein to the PEG$_{2000}$-DSPE linker increases the apparent molecular size of the scFv by approximately 3 kDa, so the reaction could be monitored by SDS-PAGE (Figure 1, lane 3). The coupling reaction routinely yields 75-95% of scFv coupled to PEG$_{2000}$-DSPE, preserving >80% of immunoreactivity as determined by BIAcore analysis (results not shown). Preservation of immunoreactivity as well as the absence of conjugates comprising more than one linker (single conjugate band on Figure 1, Lane 3) suggests that despite the presence of additional scFv cysteines involved in intramolecular V-domain disulfide bonds, the reduction conditions were specific to the thiol group engineered onto the C-terminus of the scFv, thus providing the single attachment site to PEG$_{2000}$-DSPE.

Upon incubation of antibody-PEG-DSPE conjugates with preformed liposomes, the hydrophobic DSPE domain spontaneously incorporates itself into the liposome lipid bilayer, thus "tethering" the antibody ligand to the liposome surface [25, 26]. Incubation of scFv-PEG-DSPE conjugate with commercial liposomal doxorubicin (Doxil, Alza Corp.) at 30-40 scFv/liposome overnight at 37°C resulted in 75-90% incorporation of the conjugate into drug-loaded liposomes. Similar incorporation efficiency was achieved in the range of 2-100 scFv/liposome using HPTS-loaded unilamellar liposomes of POPC, cholesterol, and PEG$_{2000}$-DSPE (3:2:0.3 molar ratio) which have lipid bilayers in the fluid

state as opposed to gel state of Doxil liposome bilayer [14, 15]. Leakage of encapsulated HPTS or doxorubicin during this process was quite low, typically less than 3% (results not shown). SDS-PAGE analysis confirmed efficient incorporation of F5 conjugate into the liposomes and complete removal of all extraliposomal proteins by subsequent gel-chromatography on Sepharose 4B (Figure 1, lane 4). The efficient separation of F5-PEG-DSPE micelles and the liposomes on the Sepharose 4B column was determined by spectrophotometrical analyses of the fractions and showed that the conjugate and the liposomes are resolved, Figure 1B.

Effect of the scFv density on antigen binding and intracellular uptake of F5-immunoliposomes.

The binding of immunoliposomes containing 2-100 scFv/liposome to ErbB2 was investigated by surface plasmon resonance (SPR) under mass transport limiting conditions [27], well below saturating concentrations of immunoliposomes. The rate of binding to ErbB2 ECD was directly proportional to the number of scFv/liposome ($r = 0.99$), as shown in Figure 2A.

The equilibrium binding constant (K_D) for the scFv antibody binding to the ErbB2 ECD was also determined by SPR. Following incubation of F5-immunoliposomes with varying amounts of soluble extracellular domain of ErbB2, the binding concentration was determined by SPR, also under mass transport limited conditions. Because the slope of the mass transport limited SPR signal of the liposome binding is directly proportional to the concentration, the K_D can be determined by fitting the data as described [27, 28]. The K_D for the monovalent binding of F5 scFv conjugated to the liposome ($K_D = 111$nM) is in

close agreement with the 160 nM K_D value of soluble, unconjugated F5 scFv for ErbB2, also determined by SPR, indicating that conjugation to the liposome does not significantly affect the monovalent interaction with the antigen. Varying the density of scFv fragments on the liposome surface from 0-30 scFv/liposome produced a dramatic increase in cellular uptake which reached a maximum uptake value at 30 scFv/liposome (or approximately 1 scFv per 1300 phosholipids in the outer leaflet of the liposome). Increasing the number of scFv from 30 to 100 per liposome did not increase uptake further (Figure 2B). This result is comparable to our previously reported result (approximately 40 Fab'/liposome) for the trastuzumab-Fab'-conjugated liposomes [2] as well as with the findings of Maruyama et al. [29] showing that 30 IgG molecules per liposome are optimal for tumor cell uptake.

Specificity of F5-immunoliposome uptake into ErbB2 expressing tumor cells

To investigate the dependence and specificity of F5-immunoliposome uptake on ErbB2 cellular expression, total cellular uptake was determined for a number of tumor cell lines with varying ErbB2 expression levels (Figure 3). For comparison, the relative antigen expression on the same cell lines was determined by flow cytometry using F5 scFv containing the FLAG tag sequence, detected with anti-FLAG FITC conjugated antibody. Overall, F5-immunoliposome uptake correlated with increasing ErbB2 expression ($r = 0.80$); uptake in cell lines having low ErbB2 expression (MDA-MB-468, MDA-MB-231, and MCF7) was two to three orders of magnitude below that of ErbB2-overexpressing cell lines (MCF7/HER2, BT474, SK-OV-3, and SK-BR-3). The specificity of the F5 immunoliposome uptake is further exemplified by its uptake into the

ErbB2-transfected and overexpressing MCF7/HER2 cells. The transfected MCF7/HER2 subline takes up two orders of magnitude more of the F5 immunoliposomes than the parental MCF7 cells that express 45-fold lower levels of ErbB2 receptor [17]. Among the ErbB2 overexpressing cell lines, however, total F5 immunoliposome uptake correlated poorly ($r = 0.56$) with the magnitude of ErbB2 overexpression. For example, SK-OV-3 cells accumulated less than half the total amount of F5 immunoliposomes compared to SK-BR-3 cells, despite comparable levels of ErbB2 overexpression (Figure 3). This may relate to the greater abundance of an alternatively spliced ErbB2 transcript in SK-OV-3 cells [30]. Excess extracellular domain encoded by this alternative transcript, which confers resistance to trastuzumab (Herceptin) when transfected into sensitive cell lines, is sequestered in the perinuclear Golgi and may interfere with endocytosis of surface membrane, full-length, ErbB2 [30].

Cell internalization and endosomal accumulation of F5-immunoliposomes

For fluorescence microscopy studies of the cells with F5- immunoliposomes, where the marker sensitivity to pH was undesirable, BODIPY-disulphonate (BODIPY-DS) was substituted for HPTS. BODIPY-DS loaded F5-immunoliposomes and tetramethylrhodamine-labeled transferrin were co-incubated with SK-BR-3 cells at 37°C. F5-immunoliposomes quickly entered the cells (Figure 4A). Unlike trastuzumab-Fab'-containing immunoliposomes [2], F5-immunoliposomes did not co-localize with transferrin but rather accumulated into a perinuclear compartment consistent with the late endosomes. Incubation of F5-immunoliposomes with MCF7 cells did not result in any detectable uptake. Thus F5 immunoliposomes efficiently entered cells in ErbB2

receptor-specific manner and appeared to accumulate in a late endosomal compartment. The kinetics of internalization of F5 immunoliposomes containing approximately 30 scFv per liposome determined using the pH sensitive dye HPTS [2, 13, 20] (0.017 min^{-1}; Fig 5B) was three fold higher than the internalization rate determined for the unconjugated scFv (0.0056 min^{-1}). A similar correlation has been reported for liposomes conjugated with transferrin [31].

In vivo efficacy of doxorubicin delivered by F5-immunoliposomes

Using a xenograft model of a human ErbB2 overexpressing breast cancer (BT474) [32] we compared the antitumor efficacy of doxorubicin-loaded F5-immunoliposomes derived from a commercial preparation of sterically stabilized liposomal doxorubicin (Doxil) with that of parental (non-targeted) Doxil. In animals with large (350-400 mm^3) subcutaneous tumors, three weekly i.v. injections of F5-immunoliposomal doxorubicin (5 mg/kg) produced substantial tumor regressions (Figure 5A). After second and third treatments, tumor regressions in the F5 targeted group were significantly superior to non-targeted Doxil ($p = 0.001$ by two-tailed non-paired Student's t-test) and far superior to the control PBS treatment (Figure 5B).

Discussion

Receptor mediated endocytosis is an essential first step for many antibody targeted therapeutic approaches, including immunotoxins, immunoliposomes, antibody-drug conjugates and antibody targeted gene delivery (reviewed in ref. [33]). Since the efficiency of antibody mediated endocytosis varies considerably depending on the antigen and epitope recognized [34, 35], it typically has been necessary to screen for antibodies capable of mediating cell internalization by individual labeling of antibodies or antibody fragments. We recently developed a method to directly select internalizing antibodies from phage libraries [7] by panning on a target cell line. Selection is not directed at a specific cell surface receptor, but rather the panel of receptors capable of internalization. Using this approach, a panel of antibodies were selected from a naïve phage library that internalized into the breast tumor cell line SK-BR-3 [9]. Here we demonstrate that such antibodies can be used to generate a targeted therapeutic with significant anti-tumor activity in vivo.

For construction of the antibody targeted therapeutic agent we selected the F5 scFv that binds the ErbB2 growth factor receptor tyrosine kinase. ErbB2 (HER2/*neu*) is a proto-oncogene, that is overexpressed in 20-30% of human breast carcinomas as well as in gastric, lung, colon, ovarian and pancreatic adenocarcinomas (for review, see ref. [36]). ErbB2 represents a therapeutic target for antibody-mediated drug delivery as it undergoes endocytosis in response to antibodies binding certain extracellular epitopes [2, 6, 35]. In addition, the clinical relevance of ErbB2 as a cancer target has been validated by the recent FDA approval of the anti-ErbB2 antibody trastuzumab for breast cancer therapy.

We chose to use the F5 scFv to target doxorubicin containing liposomes. Liposomes are attractive vehicles for drug encapsulation since they can carry large amounts of drug and provide protection from degradation in the circulation. A number of recent advances in liposome technology have led to the optimization of liposomal drug carriers for effective anti-cancer treatment *in vivo* (for review, see [37]). For example, steric stabilization by coating liposomes with polymers such as polyethylene glycol (PEG) greatly increases their circulation time due to increased resistance to clearance by the mononuclear phagocyte system (MPS), thus facilitating selective extravasation in solid tumors [38, 39]. The sterically stabilized liposomes do not directly enter the tumor cells but accumulate within the tumor interstitium where the drug then passively diffuses into the tumor cells [37, 40]. Therapeutic efficacy can be improved by delivering the liposomes directly into the tumor cells. Indeed, anti-ErbB2 immunoliposomes containing doxorubicin and targeted by the Fab' fragment of trastuzumab are endocytosed by ErbB2 expressing cells and have shown greater therapeutic efficacy against ErbB2-overexpressing xenografts than liposomal doxorubicin in the absence of targeting, although targeting does not increase the overall tumor uptake of liposomal drug [2, 41]. The degree of benefit from liposomal targeting appears to depend to some extent on the degree of cellular internalization of the immunoliposome upon receptor binding. Likewise, lack of immunoliposome internalization following receptor binding is associated with poor cytotoxicity and lack of therapeutic advantage [42-45].

To investigate the potential for tumor targeting by scFv selected for internalization, the F5 scFv was engineered for coupling to liposomes. A C-terminal cysteine was engineered into the F5 scFv molecule and a high level expression vector

constructed using the phoA promoter and kanamycin resistance. The resulting expression system is tightly regulated, easily scalable to larger fermentation vessels, and uses a resistance marker compatible with clinical good manufacturing practice (cGMP). Since the F5 scFv contains a V_H gene derived from the human V_H3 family, it binds protein A. This allows the elimination of epitope tags frequently used for purification, such as hexahistidine, which could pose a regulatory hurdle. These vector and scFv gene modifications were incorporated to facilitate pharmaceutical production of F5 immunoliposomes and ensure the possibility of future clinical translation.

F5 scFv coupling to prefabricated liposomes first required its conjugation via the engineered C-terminal cysteine's free thiol to the maleimide group formed at the free end of a PEG-DSPE linker. Use of a PEG linker prevents neighboring liposomal PEG molecules from interfering with antibody binding of the cell surface receptor [2], thus the affinity of the liposome-conjugated F5 was essentially equal to that of the free scFv. Such thiol-reactive PEG-lipid linkers previously have been applied to construct liposomes with conjugated Fab' fragments [2] and whole antibodies [29, 46, 47]. The presence of hydrophilic PEG domain prevents precipitation of the conjugate which stays in aqueous solution presumably in a micellar form. However, the presence of a hydrophobic lipid tail results in the capture of scFv-linker conjugate into preformed liposomes by simple mixing. This afforded a single-step conversion of commercial doxorubicin-loaded liposomes into anti-ErbB2 immunoliposomes with high yield (75-90%).

In vitro, ErbB2-overexpressing cells showed specific uptake of F5-immunoliposomes into ErbB2 overexpressing cells in proportion to their endocytotic

capacity. Fluorescence of liposome-entrapped dye pointed to endosomal accumulation by the internalized F5 immunoliposomes. However, F5 immunoliposomes did not co-localize with transferrin. Rather, the F5 immunoliposomes appeared to localize into a perinuclear compartment consistent with the late endosomes.

The F5 scFv is of entirely human origin. This eliminates the need for timely and costly cloning and humanization of antibodies undertaken to evade an human anti-mouse (HAMA) immune response. Incorporation of humanized antibodies into immunoliposomes may also lead to an increased HAMA response compared to free antibody due to enhanced immunogenicity from uptake of antibody coated liposomes into reticulendothelial cells. This could result in increased opsonization and uptake into macrophages and consequently a shorter circulation time [48]. Immunogenicity should be less for completely human antibody fragments. In addition, the F5 scFv does not have growth inhibitory properties of the FDA approved anti-ErbB2 antibody, trastuzumab [49], which may be linked to the high incidence of cardiotoxicity in patients treated with trastuzumab, especially when administered along with doxorubicin [50].

In summary, we believe this approach provides a generic route for rapid development of antibody targeted drugs with potent *in vivo* anti-tumor activity. Human antibodies are selected from phage libraries for internalization into a target tumor cell line and the biology and specificity of the selected antibodies is confirmed by secondary screens. The scFv genes are subcloned into the high level expression system described here and the purified scFv conjugated to linker and immunoliposomes formed by membrane capture. If efficacy is verified in the appropriate animal model, then expression and purification can be scaled up for current good manufacturing practice

(cGMP) manufacture. For example, based on preclinical data partially described in this publication, the F5 scFv expression plasmid has been transferred to the National Cancer Institute Monoclonal Antibody and Recombinant Protein facility for expression and purification scale up and possible cGMP manufacturing. Using the membrane capture system, a single batch of cGMP antibody could be used to target liposomes carrying other anticancer pharmaceuticals or the scFv antibody may be coupled to other nanoparticle delivery systems, toxins or drugs using the conjugation chemistry described here.

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Figure legends

Figure 1. SDS-PAGE analysis of F5 scFv purification, reduction, and coupling to maleimide-PEG-DSPE and analyses of F5-PEG-DSPE insertion into liposomes. (A) Lane 1: F5 scFv purified using Protein A chromatography; Lane 2: Protein A-purified F5 scFv after reduction on a ReduceImm column; Lane 3: F5 scFv after conjugation to maleimido-PEG-DSPE; lane 4: F5 scFv after incorporation into liposomes and purification of immunoliposomes by gel filtration on Sepharose 4B. (B) F5-PEG-DSPE or doxorubicin liposome (Doxil) were applied onto a Sepharose 4B column and eluted with HEPES-buffered saline. Fractions were collected and spectrophotometrically analyzed for F5 protein (absorbance at 280 nm) or doxorubicin (absorbance at 485 nm). The amounts of F5 or doxorubicin per fractions were expressed as % of total applied. The arrow marks the column total volume (V_0).

Figure 2. Effect of scFv density on the binding of immunoliposomes to ErbB2 and on the uptake by SK-BR-3 cells. (A) Effect of scFv density on immunoliposome binding to the ErbB2 extracellular domain as determined by surface plasmon resonance. Immunoliposomes were formed by incorporation of varying amounts of scFv-PEG-DSPE conjugate into preformed 100 nm POPC/Cholesterol/PEG-DSPE liposomes and analyzed in a BIAcore 1000 instrument. Measurements were made at an immunoliposome concentration of 50 μM of liposome phospholipid; (B) Effect of scFv density on the total uptake of anti-ErbB2 immunoliposomes by SK-BR-3 cells. Immunoliposomes were incubated with the cells at 100-fold excess over total cellular ErbB2 receptors for 12 hr

and concentration of 50 μM of liposome phospholipid. Error-bars represent the standard deviation of triplicate experiments.

Figure 3. Effect of cell surface ErbB2 expression as determined by flow cytometry on the total cellular uptake of anti-ErbB2 immunoliposomes (A) Total uptake of F5 immunoliposomes after 12 hours of incubation; (B) ErbB2 expression level as determined by flow cytometry with the F5 scFv detected by anti-FLAG FITC. Error-bars represent the standard deviation of triplicate experiments.

Figure 4. Binding and endocytosis of F5 immunoliposomes by ErbB2-overexpressing tumor cells. (A) Immunofluorescence analysis of F5-liposomes loaded with BODIPY (yellow/green) and transferrin-tetramethylrhodamine (red) after two hours of co-internalization into SK-BR-3 cells; (B) Kinetics of anti-ErbB2 immunoliposomes uptake by SK-BR-3 cells at 200 μM of liposome phospholipid. Depicted are the internalized fraction *(closed circles)* and surface bound fraction *(closed triangles).*

Figure 5. Efficacy of anti-ErbB2 immunoliposomes in a human ErbB2 overexpressing breast cancer model. (A) Anti-ErbB2 immunoliposome-Doxil containing the F5 scFv *(triangles)* were administered i.v. on days 18, 25, and 32 (arrows) at a total doxorubicin dose of 15 mg/kg as indicated in the text. The control treatment group was treated with Doxil at the same doxorubicin dose *(circles).* (B) Anti-ErbB2 immunoliposome-Doxil containing the F5 scFv *(triangles)* administered on days 21, 28, and 35 (arrows, same dose as above) are compared to control (PBS) treatment *(open circles).* Data represent mean tumor volumes; mm^3+/- SE.

Figures

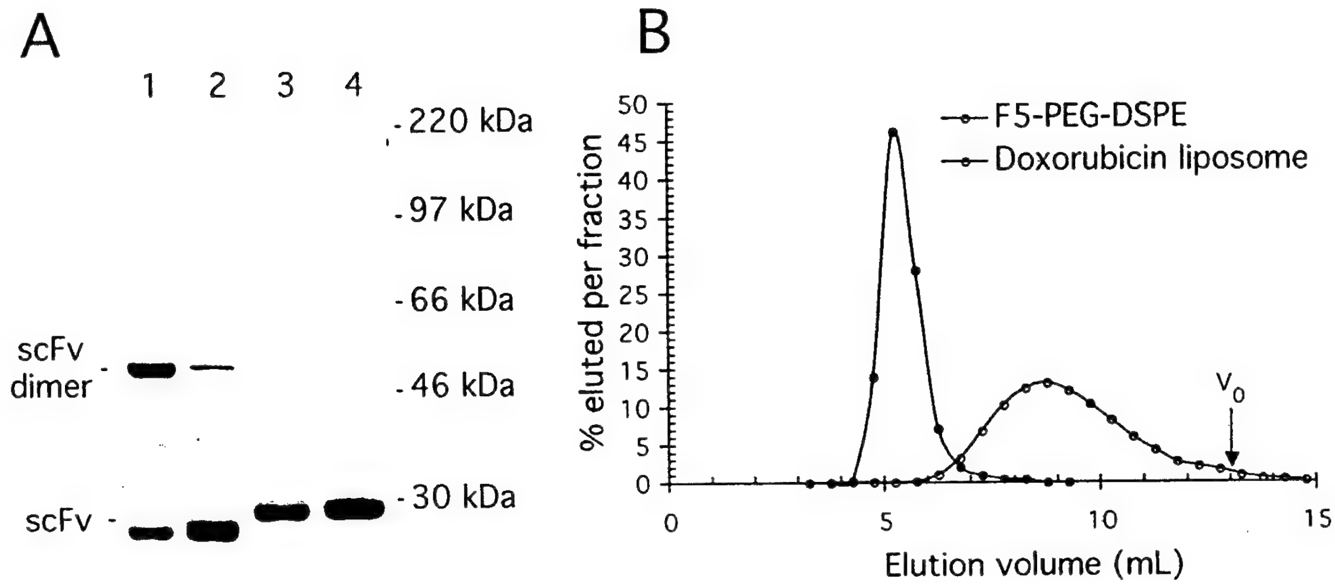
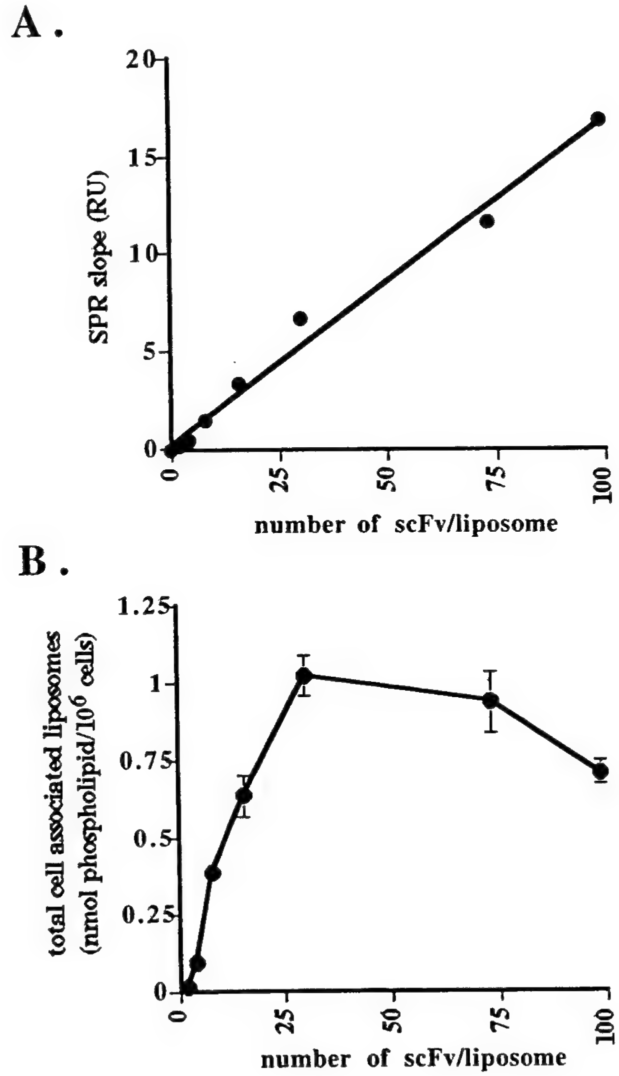


Figure 1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SPR slope (RU)
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15
10
5
0
0
25
50
75
100
number of scFv/liposome
B.
total cell associated liposomes
(nmol phospholipid/10^6 cells)
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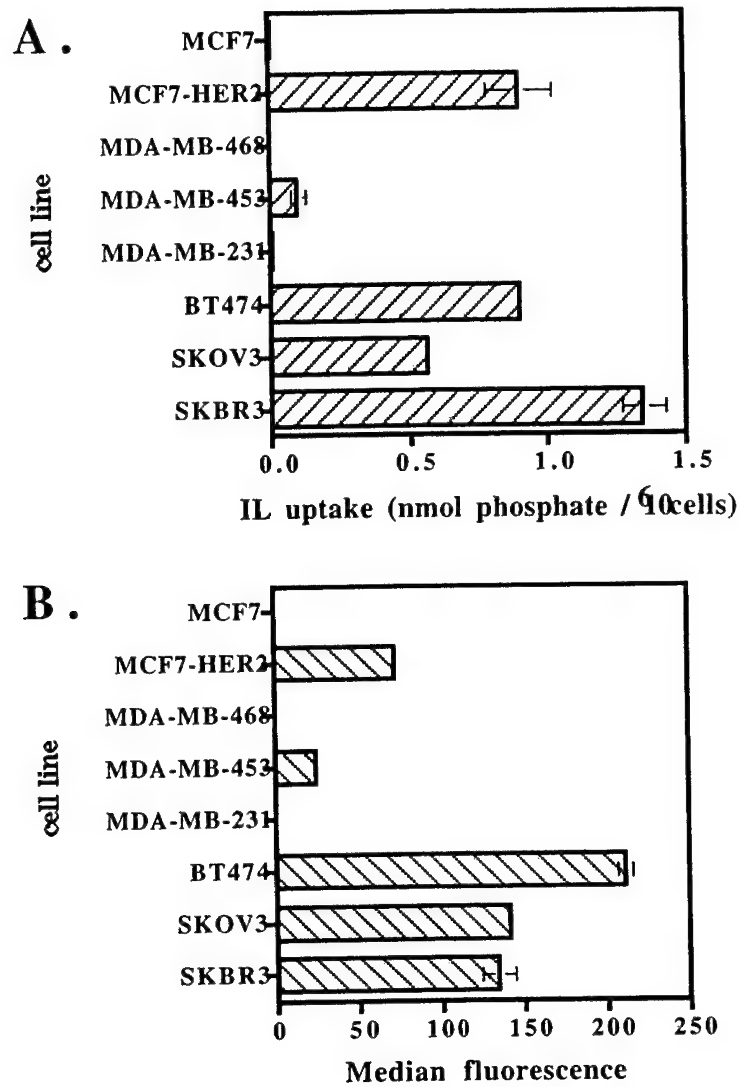
0.75
0.5
0.25
0
0
25
50
75
100
number of scFv/liposome

Figure 2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MCF7
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MDA-MB-468
MDA-MB-453
MDA-MB-231
BT474
SKOV3
SKBR3
cell line
0.0
0.5
1.0
1.5
IL uptake (nmol phosphate / 10^6 cells)
B.
MCF7
MCF7-HER2
MDA-MB-468
MDA-MB-453
MDA-MB-231
BT474
SKOV3
SKBR3
cell line
0
50
100
150
200
250
Median fluorescence

Figure 3

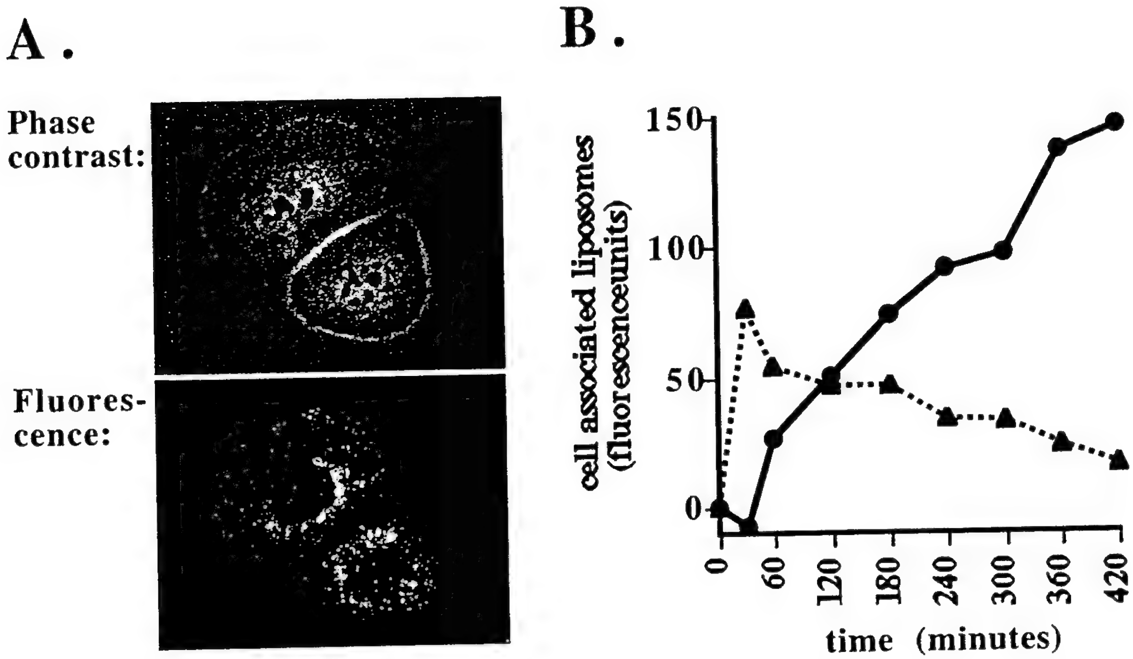
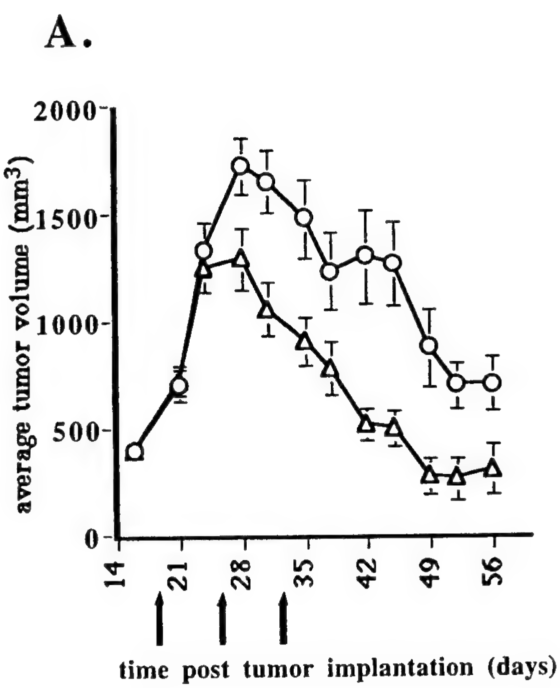

A.
Phase contrast:
Fluorescence:
B.
cell associated liposomes (fluorescenceunits)
150
100
50
0
0
60
120
180
240
300
360
420
time (minutes)

Figure 4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B.
average tumor volume (mm³)
0
500
1000
1500
2000
0
1000
2000
3000
4000
5000
14
21
28
35
42
49
56
time post tumor implantation (days)

Figure 5

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Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library

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Abstract

The first step in developing a targeted cancer therapeutic is generating a ligand that binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. For this work, we generated human monoclonal antibodies to the EGF receptor (EGFR), an antigen overexpressed on many solid tumors. Single chain Fv (scFv) antibody fragments were directly selected by panning a phage display library on tumor cells (A431) overexpressing EGFR or Chinese hamster ovary cells (CHO/EGFR cells) transfected with the EGFR gene and recovering endocytosed phage from within the cell. Three unique scFvs were isolated, two from selections on A431 cells and two from selections on CHO/EGFR cells. All three scFv bound native receptor as expressed on a panel of tumor cells and did not bind EGFR negative cells. Phage antibodies and multivalent immunoliposomes constructed from scFv were endocytosed by EGFR expressing cells as shown by confocal microscopy. Native scFv primarily stained the cell surface, with less staining intracellularly. The results demonstrate how phage antibodies binding native cell surface receptors can be directly selected on overexpressing cell lines or transfected cells. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences. Depending upon the format used, the antibodies can be used to deliver molecules to the cell surface or intracellularly.

Keywords: Receptor mediated endocytosis; Epidermal growth factor receptor; Phage antibody library; Single chain Fv; scFv; Tumor targeting

1. Introduction

Traditional cancer therapies have relied on the differential toxicity of chemotheraputic agents on tumor cells compared to normal cells. Recently, improved understanding of the molecular basis of cancer makes possible the development of therapies with increased efficacy and reduced toxicity. Studies of tumorigenesis have identified cell surface receptors which are either tumor or lineage specific, such as CD20 (Einfeld et al., 1988) and mutant forms of epidermal growth factor receptor (EGFR) (Garcia de Palazzo et al., 1993) or receptors which are over-

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expressed in tumors, such as ErbB2 (Slamon et al., 1989). Such receptors can be targeted with antibodies to allow specific drug interaction with only the tumor cell. In some instances, binding of "naked" antibody to the tumor cell can cause growth inhibition (Carter et al., 1992) or apoptosis (Ghetie et al., 1997; Taji et al., 1998). Alternatively, the antibody can be used to deliver a "toxic payload" to the cell. Toxic mechanisms include activation of the immune system, e.g., with bispecific antibodies, fusions to co-stimulatory molecules, or fusion with a toxic payload including radioisotopes, chemotherapeutics, toxins, or genes. For some strategies, it is necessary for the antibody to remain on the cell surface (e.g., bispecific therapies). For other approaches, it is necessary that the antibody deliver its payload into the cytosol (e.g., immunotoxins and gene therapy). In both cases, antibody recognition of the native receptor as expressed on the cell surface is required.

Phage antibody libraries have become an important source for the development of completely human therapeutic antibodies (Marks and Marks, 1996; Marks et al., 1991) to a wide range of antigens including tumor growth factor receptors (Schier et al., 1995). Antibodies generated from phage libraries have typically been selected using purified antigens or peptides immobilized on artificial surfaces. This approach may select antibodies that do not recognize the native protein in a physiologic context, as on the surface of cells. Attempts have been made to select on antigen in native conformation using cell lysates (Parren et al., 1996; Sanna et al., 1995; Sawyer et al., 1997) fixed cells (Van Ewijk et al., 1997) or living cells (Andersen et al., 1996; Cai and Garen, 1995; de Kruif et al., 1995; Marks et al., 1993; Siegel et al., 1997). The few successful selections performed on such heterogeneous material were generally done using small libraries from immunized sources. The use of immunized libraries limits the spectrum of antigen specificities that can be potentially obtained from the same library and typically yields murine antibodies. Selection of binders from large naïve libraries by cell panning is greatly limited by high background binding of non-specific phage and relatively low binding of specific phage (Pereira et al., 1997; Watters et al., 1997; Becerril et al., 1999).

Using a model system and an ErbB2 phage antibody we recently demonstrated that phage antibodies binding internalizing surface receptors can be endocytosed by mammalian cells and recovered in infectious form from within the cell (Becerril et al., 1999). Enrichment of ErbB2 phage over non-specific phage was 10–30 times higher when phage were recovered from within the cell compared to recovery from the cell surface, suggesting that cell selection specificity could be increased by recovering internalized phage. We confirmed this by applying this methodology to generate a panel of anti-tumor antibodies which were endocytosed into the breast tumor cell line SKBR3 as well as other tumor cells (Poul et al., 2000). Two of the specificities isolated included ErbB2 and transferrin receptor antibodies.

For this work, we applied the methodology to generate EGFR antibodies which recognized the native receptor on cells and could be used for tumor targeting. EGFR is overexpressed in many carcinomas (Baselga and Mendelsohn, 1994; Chrysogelos and Dickson, 1994; De Jong et al., 1998; Harris, 1994; LeMaistre et al., 1994) and can be exploited to differentiate and target cancer cells from normal cells. For selections, two cell lines were used as the source of antigen: a transfected Chinese hamster ovary cell (CHO/EGFR) and EGFR-overexpressing cancer cell line A431. The results indicate the generality of the approach and its usefulness in generating antibodies to known receptors in the absence of purified recombinant protein.

2. Materials and methods

2.1. Cell culture

CHO cells stably transfected with EGFR full length receptor (Morrison et al., 1993) (CHO/EGFR) were grown in F12 selective media (G418, Mediatech, 0.8 g/l) supplemented with 10% fetal calf serum (FCS). The parent cell line (CHO) was grown in non-selective F12 complete media supplemented with 10% FCS. A431 cells were grown in DMEM supplemented with 10% FCS. MDA-MB-453 and MDA-MB-468 cells were grown in Leibovitz media supplemented with 10% FCS in the absence of CO_2. All other cell lines were grown at 37°C in the presence of 5% CO_2.

2.2. Phage antibody selections

2.2.1. Selections on CHO/EGFR cell monolayer

CHO/EGFR cells grown on a 10-cm plate at 80–90% confluence were incubated with 1 ml of phage antibody library (5×10^{12} cfu/ml) (Sheets et al., 1998) in the presence of 2×10^{6} CHO cells in complete media (3 ml) for 1.5 h at 4°C. CHO cells were used to deplete the library of non-specific clones. The supernatant was aspirated and cells were washed six times in cold complete media for 10 min per wash. Receptor internalization was induced by addition of pre-warmed (37°C) complete media and incubation at 37°C, 5% CO_2 for 15 min. This time period has been shown to be appropriate for the observation of EGFR internalization (Vieira et al., 1996). After internalization, non-internalized cell-membrane bound phage were eluted by washing cells on the plate with cold glycine buffer (50 mM glycine, 150 mM NaCl, 200 mM urea, 2 mg/ml polyvinylpyrrolydone, pH 2.8) three times for 10 min per wash at 4°C. Immobilized cells were washed 1× in complete media. The internalized phage were recovered by removing cells in trypsin and washing in complete media. Cells were pelleted by centrifugation at 1000 rpm, lysed in 0.5 ml 100 mM triethylamine (TEA) for 10 min and neutralized in 1 ml 1 M Tris, pH 7.

2.2.2. Selections on A431 cells in suspension

A431 cells growing on a 15-cm culture dish (90% confluence) were removed in 2 mM EDTA–phosphate-buffered saline (PBS) and washed twice in cold PBS (25 ml). To deplete the library of non-specific phage, 5×10^{6} fibroblast cells (ATCC, CRL1634) were incubated with 1 ml of phage antibody library in 3 ml complete media (DMEM–10% FCS) for 1 h rocking at 4°C. Fibroblast cells were pelleted by centrifugation at 1000 rpm and the supernatant was recovered. A431 cells were incubated in a 15-ml culture tube with the depleted phage antibody library (supernatant from the previous step) for 1.5 h rocking at 4°C. Cells were subsequently washed 10 times in cold complete media. Cells were incubated for 30 min at 37°C in pre-warmed complete media to allow receptor internalization. Non-internalized phage were removed from the cell surface by 10 washes in cold PBS and a final wash in glycine buffer. Cells were lysed immediately, following a single glycine wash, in 0.5 ml 100 mM TEA and neutralized in 1 ml 1 M Tris, pH 7.

2.3. Phage rescue, preparation and titration

Phage were titered by infection of eluted phage into *Escherichia coli* TG1 (Marks et al., 1991). Phage were prepared for the next round of selection by infection of *E. coli* TG1 with eluted phage and rescue with VCS-M13 (Stratagene) helper phage as previously described (Marks et al., 1991). After overnight growth at 30°C, phage were purified and concentrated from bacterial supernatant with polyethylene glycol 8000 (PEG8000) (Marks et al., 1991) and resuspended in 1.5 ml PBS for use in the next round of selection or for use in flow cytometry. For each cell type, a total of three rounds of selection were performed.

2.4. Polyclonal phage enzyme-linked immunosorbent assay (ELISA)

EGFR-ECD was expressed in CHO cells and purified by concavalin A agarose (Vector Laboratories) affinity chromatography. Ninety-six-well microtiter plates (Falcon, 353912) were coated overnight at 4°C with 10 μg/ml of EGFR-ECD in PBS. Plates were washed three times with PBS and 50 μl of 1.0×10^{11} cfu/ml of polyclonal phage in PBS buffer (prepared as described in Section 2.3) was added to each well and incubated for 1 h. Wells were washed three times with PBS containing 0.1% Tween 20 (TPBS) and three times with PBS. Binding of phage antibodies was detected with peroxidase-conjugated anti-M13 antibody (Amersham-Pharmacia) diluted 1:1000 in PBS and ABTS (Sigma) as substrate.

2.5. Evaluation of polyclonal phage mixtures by flow cytometry

Polyclonal phage were screened for binding to whole cells by fluorescence activated cell sorting (FACS) analysis. EGFR expressing cell lines MDA-MB-468 and CHO/EGFR were used to identify EGFR binding antibodies and EGFR negative cell lines MDA-MB-453 and CHO were used to de-

termine specificity. Cells were grown to 80–90% confluence and removed in 2 mM EDTA–PBS. Cells were counted and washed once in cold PBS and twice in FACS buffer [cold 0.5% bovine serum albumin (BSA) (fraction V, Sigma)–PBS]. Cells were placed in FACS tubes (100 000 cells/well) and incubated with polyclonal phage (50 $\mu l/10^{12}$ cfu/ml) for an hour on ice. Cells were washed twice in FACS buffer and incubated with α-M13-biotin (Amersham-Pharmacia, 1:5000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and incubated with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and then analyzed by FACS on the PE channel. Fluorescence was measured in a FACSort™ (Beckton Dickinson) and mean fluorescence was calculated using the Cellquest™ software.

2.6. Isolation and characterization of monoclonal EGFR antibodies

To facilitate subsequent purification of soluble single chain Fv (scFv), the polyclonal scFv gene population from the third round of selection was subcloned in batch into the expression vector pUC119mycHis (Schier et al., 1995) resulting in the addition of a c-myc epitope tag and hexahistidine tag at the C-terminus of the scFv. Briefly, phagemid DNA was prepared from the third round of selection, the scFv genes excised using the restriction enzymes *Sfi*1 and *Not*1, and the gene repertoire gel purified and ligated into pUC119mycHis digested with *Sfi*1 and *Not*1. After transformation of *E. coli* TG1, single ampicillin resistant colonies were packed into 96-well microtiter plates and scFv expression induced by the addition of IPTG as previously described (Schier et al., 1996). Bacterial supernatant containing scFv was used directly for ELISA. For EGFR-ECD ELISA using unpurified scFv, microtiter plates (Falcon) were coated with 10 μg/ml EGFR-ECD in PBS overnight at 4°C. Plates were incubated with bacterial supernatants at room temperature for 1 h and then washed once in TPBS and twice in PBS. Protein binding was detected with anti-myc tag antibody 9E10 followed by incubation in secondary antibody anti-mouse-horseradish peroxidase (HRP) (Sigma) for 30 min as previously described (Schier et al., 1996). Following a final set of washes, binding was detected with ABTS substrate. For further studies of monoclonal scFv, expression from pUC119 mycHis was scaled up into 500-ml cultures in 2 L culture flasks. Cultures were grown and scFv expressed (De Bellis and Schwartz, 1990) as previously described (Schier et al., 1996). scFv was harvested from the bacterial periplasm by osmotic shock (Breitling et al., 1991) and purified by immobilized metal affinity chromatography (Hochuli, 1988) using a Ni-NTA column (Qiagen) and gel filtration, as previously described (Schier et al., 1996).

2.7. Covalent labeling of α-EGFR scFv with fluoroisothiocyanate (FITC)

A 1-ml (1 mg) volume of E12 scFv was dialyzed against 50 mM carbonate buffer, pH 8.5 overnight. Fluoroisothiocyanate (FITC) labeling reagent, 6-(fluorescein-5-[and-6]-carboxamido)hexanoic acid, succinimidyl ester [5(6)-SFX, Molecular Probes] was dissolved in dimethylsulfoxide (DMSO) or dimethylformide (DMF) (5–10 mg/ml) and added to the scFv at a volume:volume ratio of 1:20. The reaction was conducted for 1 h at room temperature. Free labeling reagent was separated from labeled antibody on a S-25 gel filtration column (Sephadex). E12 scFv was incubated with CHO cells and CHO/EGFR cells and no background binding due to free label could be detected. No fluorescence shift was detected for CHO cells stained with the labeled antibody. The fluorescence shift detected on CHO/EGFR cells was therefore wholly attributed to the antibody–receptor interaction.

2.8. Microscopy

2.8.1. Fixed cell microscopy

Confirmation of α-EGFR phage antibody internalization was obtained by confocal microscopy. Cells were grown on coverslips in 24-well plates and at 80% confluence were incubated with phage antibody (10^{10} cfu/ml, in fresh complete media) for 2 h at 37°C. Plates were placed on ice to halt receptor internalization and the coverslips washed 10 times in cold PBS (1 ml per wash). Cells were then washed three times for 10 min in cold glycine buffer, pH 2.8

(50 mM glycine, 150 mM NaCl) to remove surface bound antibody. Cells were washed twice in cold PBS, fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized in cold methanol for 10 min at room temperature. Coverslips were incubated with α-M13-biotin (5′–3′, 1:2000 dilution in 0.5% BSA–PBS) for 30 min followed by two washes in complete media. Phage were detected by incubation with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min while followed by two washes in complete media. Coverslips were mounted on microscope slides and 2–3 μl Vectorshield (Vector Laboratories) was applied to each coverslip to preserve fluorescence upon irradiation.

2.8.2. Live cells

scFv-mediated internalization of FITC-labeled soluble-native scFv (100 μg/ml) or fluorescent immunoliposomes was measured on live cells grown on coverslips. E12 scFv immunoliposomes were constructed as previously described and contained on average 25 scFv/liposome (Park et al., 1998). Cells were plated and grown overnight on coverslips to 80% confluency. Cells were incubated with 100 μg/ml FITC-labeled (0.5 ml volume) or unlabeled scFv or with 10 μM immunoliposomes for 2 h. Cells were washed with PBS, coverslips removed and mounted onto microscope slides for imaging. Images were collected immediately using a Leica TCS NT confocal laser fluorescence microscope with digital camera (Leica, Deerfield, IL, USA).

2.9. Affinity measurement on whole cells by FACS

Cells (A431, MDA-MB-468, MDA-MB-453) were grown to 90% confluence in DMEM (A431) and Leibovitz (MDA-MB) media supplemented with 10% FCS. Cells were harvested in 2 mM EDTA–PBS. scFv was incubated with 2.5×10^5 cells for an hour at varying concentrations (50 nM–2 μM). Cell binding was performed on ice in PBS containing 0.25% BSA in a total volume of 100 μl. After two washes in PBS–BSA (250 μl), cells were incubated with saturating amounts of anti-myc 9E10 for 30 min followed by two washes in PBS–BSA. Bound scFv was detected by staining with saturating amounts of anti-mouse FITC, Fc specific (1:200 dilution, Sigma). After a 30 min incubation, cells were washed twice and resuspended in PBS containing 1% paraformaldehyde. Determination of the binding affinity was determined using a flow cytometry based assay as previously described (Benedict et al., 1997).

3. Results

3.1. Selection of EGFR antibodies

For selections, phage were prepared from a 7.0×10^9 member human scFv phage antibody library (Sheets et al., 1998). To generate antibodies binding EGFR, phage was selected on the A431 cell line which overexpresses EGFR and on CHO cells transfected with the EGFR gene (CHO/EGFR). For selections on CHO/EGFR cells, the library was depleted of antibodies binding common cell surface receptors by adding phage to CHO/EGFR cells grown adherent to subconfluency with untransfected CHO cells in suspension. After 1 h at 4°C, CHO cells were removed from the culture flask and warm media at 37°C added to allow internalization into the target CHO/EGFR cells. For selections on A431 cells, which grow in suspension, the phage library was pre-depleted of antibodies binding common cell surface receptors by incubation with fibroblast cells. After 1 h at 4°C, the fibroblast cells were removed by centrifugation and the phage added to A431 cells in suspension at 4°C to allow binding followed by incubation at 37°C to allow phage internalization. After phage endocytosis, cells were extensively washed and then lysed with TEA. The cell lysate containing the internalized phage was used to infect *E. coli* to prepare phage for the next round of selection. Three rounds of selection were performed with the efficiency of selection monitored by titering the number of phage recovered from the cell lysate. For selections on both A431 and CHO/EGFR cells, the titer of phage recovered increased with each round of selection, consistent with enrichment for cell binding antibodies (Table 1).

3.2. Analysis of polyclonal phage for EGFR binding by ELISA and FACS

To evaluate the success of selections, polyclonal phage was prepared after each round of selection and

Table 1
Results of selection of α-EGFR scFv on whole cells in three rounds

Round	A431		CHO/EGFR	
	Phage titer (cfu)	Frequency of positives	Phage titer (cfu)	Frequency of positives
R1	1×10^{4}	ND	2×10^{3}	ND
R2	8×10^{5}	ND	8×10^{4}	ND
R3	8×10^{6}	10/94	5×10^{6}	4/282

analyzed for binding to recombinant EGFR-ECD by ELISA (Fig. 1). A signal significantly greater than background binding was observed after three rounds of selection on both A431 and CHO/EGFR cells (Fig. 1). No significant binding above background was observed after one or two rounds of selection on either cell type. Binding of polyclonal phage from the third round of selection to cell lines expressing different quantities of EGFR was studied further by flow cytometry. Phage selected on A431 cells showed a significantly greater fluorescent shift on CHO/EGFR cells than on CHO cells (Fig. 2 left panels) and on the high EGFR expressing tumor cell line MDA-MB-468 vs. the EGFR negative tumor cell line MDA-MB-453 (Fig. 2 left panels). For phage selected on CHO/EGFR cells, no significant difference in fluorescent shift was observed for binding to CHO/EGFR cells vs. CHO cells (Fig. 2 right panels). The strong shift on both cell lines indicates that the majority of the phage bind antigens common to CHO cells. Analysis of these phage for binding to high EGFR expressing MDA-MB-468 cells compared to EGFR negative MDA-MB-453 cells indicates, however, the presence of a relatively small number of phage binding EGFR (Fig. 2, right panels).

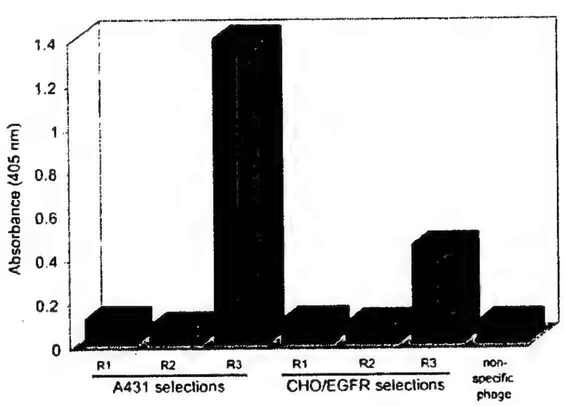

Fig. 1. Binding of polyclonal phage to recombinant EGFR as determined by ELISA. Phage was prepared from the first, second and third round of selections and analyzed for binding to recombinant EGFR by ELISA. After the third round of selection, binding was observed for selections performed on A431 cells and for selections performed on CHO/EGFR cells.

3.3. Isolation and characterization of monoclonal EGFR antibodies

Based on the ELISA and flow cytometry data indicating the presence of EGFR phage antibodies, individual clones were picked into 96-well microtiter plates and expression of native soluble scFv induced. Bacterial culture supernatants containing scFv were analyzed by ELISA for their ability to bind recombinant EGFR-ECD. For the third round of selection on A431 cells, 10/94 clones (11%) bound EGFR-ECD, while for the third round of selection on CHO/EGFR cells, 4/282 (1%) clones bound EGFR-ECD (Table 1). The relative proportions of binders is consistent with the ELISA and flow cytometry analysis of the polyclonal phage. To determine the number of unique antibodies, the scFv gene of all EGFR-ECD binding clones was analyzed by BstN1 fingerprinting followed by DNA sequencing. Two unique EGFR antibodies (E12 and B11) were isolated from selections on A431 cells. For selections on CHO/EGFR cells, the B11 scFv was re-isolated along with another unique scFv (C10).

To determine whether the monoclonal antibodies bound native EGFR as expressed on cells, phage and native scFv were prepared from each of the three

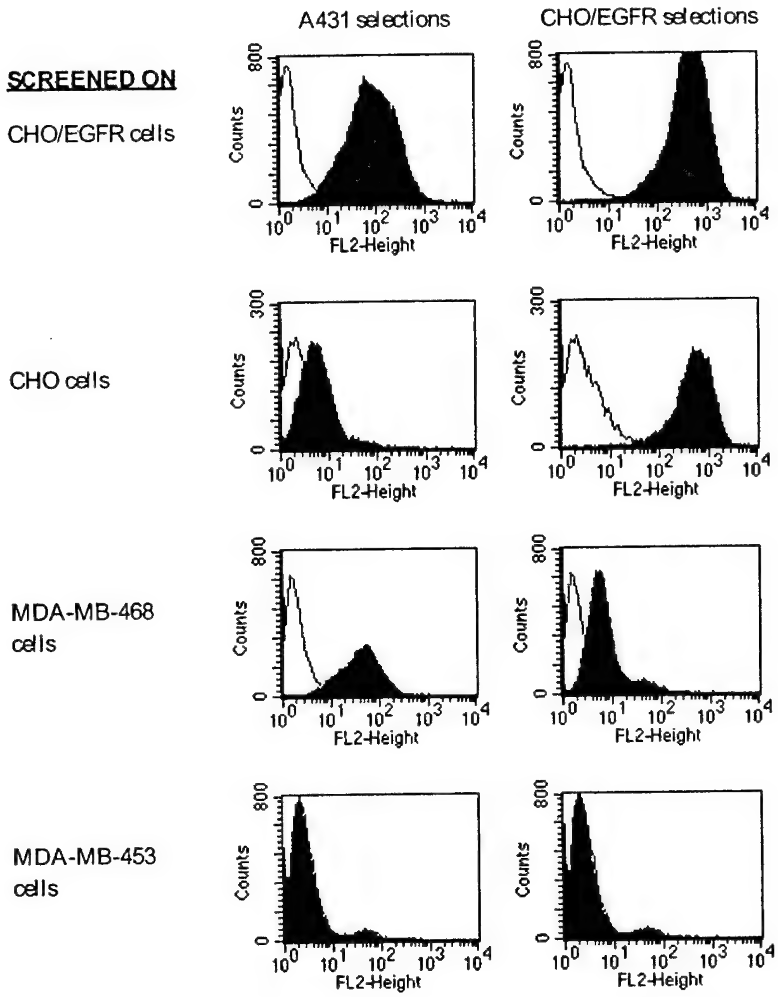

Fig. 2. Binding of polyclonal phage to EGFR expressing cells. Phage was prepared from the third round of selections performed on A431 cells and CHO/EGFR cells, and binding to a panel of cells was analyzed by flow cytometry. For selections performed on A431 cells (left panels), phage stained EGFR expressing cells (CHO/EGFR and MDA-MB-468 cells) more strongly than EGFR negative cells (CHO and MDA-MB-453 cells). For selections performed on CHO/EGFR cells (right panels), no difference in staining was observed between CHO/EGFR cells and CHO cells, however EGFR expressing cells stained more intensely than EGFR negative cells. This result suggests that many antibodies were selected that bind antigens common to CHO cells with a minority of antibodies binding EGFR.

unique scFvs and used to stain cells which were analyzed by flow cytometry. Each of the monoclonal antibodies stained EGFR expressing cells (A431, MDA-MB-468 and CHO/EGFR) but not EGFR negative cells (MDA-MB-453 and CHO) both as phage antibodies (Fig. 3) and as native scFv (Fig. 4). The binding constant for EGFR of each of the native scFvs was determined on A431 cells and on MDA-MB-468 cells (for the E12 scFv). The K_D values ranged between 217 and 300 nM (Table 2).

3.4. Cell binding and internalization of phage antibodies and scFv

Since the phage antibodies were selected on the basis of internalization, we examined the ability of

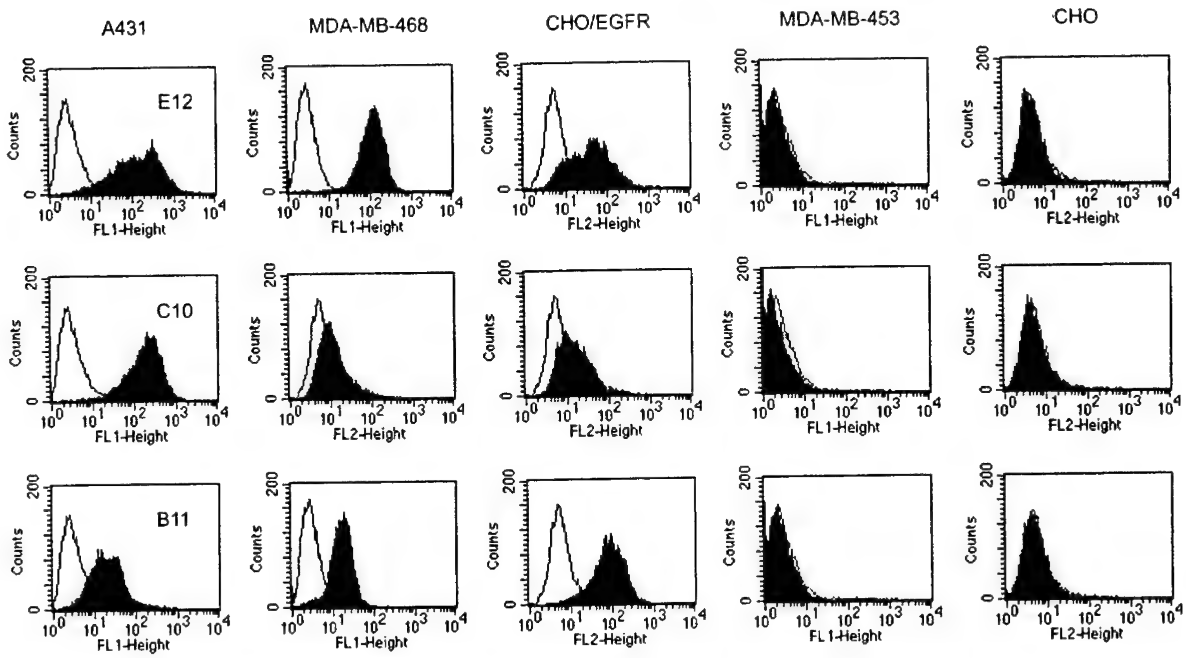

Fig. 3. Binding of monoclonal phage to EGFR positive and negative cell lines. Phage antibodies (E12, C10 and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

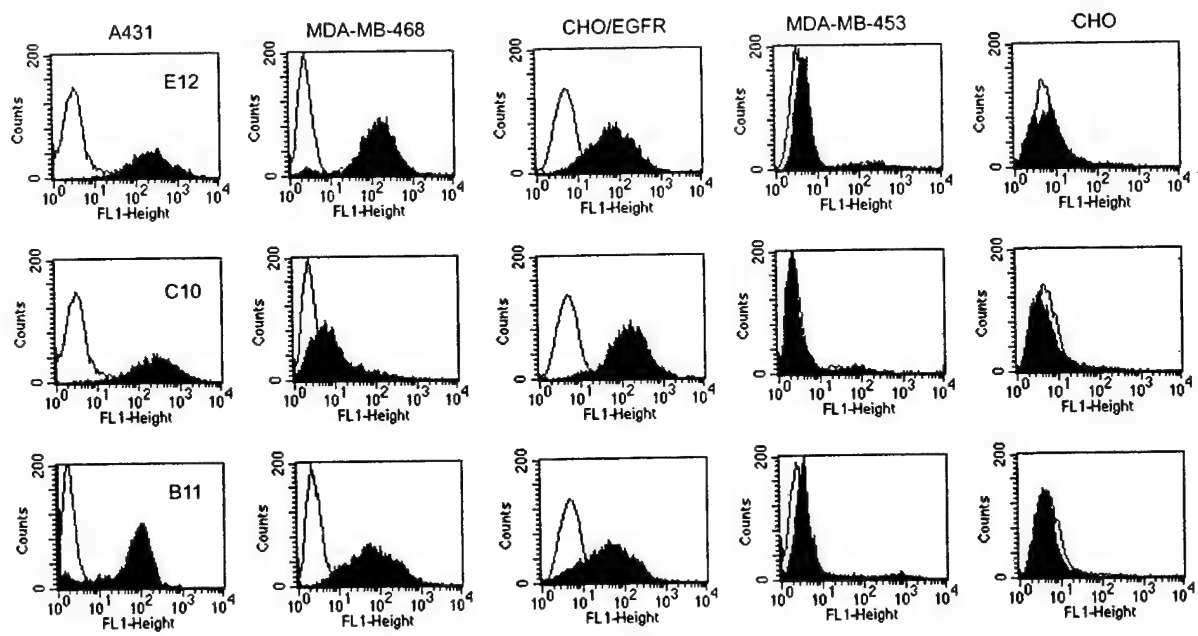

Fig. 4. Binding of monoclonal scFv to EGFR positive and negative cell lines. Purified scFvs (E12, C10, and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

Table 2
Binding affinity of α-EGFR scFv

scFv	K_D (nM)	
	A431	MDA-MB-468
E12	300	265
C10	217	–
B11	280	–

the phage antibodies to be endocytosed by EGFR expressing cells. After incubation of EGFR expressing and EGFR negative cells with phage antibodies, cells were fixed, surface phage removed with low-pH glycine and intracellular phage detected with anti-M13 antibody and confocal microscopy. Intracellular phage were detected in EGFR expressing cells (e.g., A431, MDA-MB-468 and CHO/EGFR) but not in EGFR negative cells (MDA-MB-453 and CHO). Representative results are shown for the E12 scFv on CHO/EGFR and CHO cells (Fig. 5) and on the tumor cell lines MDA-MB-468 and MDA-MB-453 (Fig. 6). To determine if native scFv were endocytosed by EGFR expressing cells, scFv were directly FITC labeled and incubated with live cells. After incubation, cells were analyzed directly by confocal microscopy allowing observation of surface bound and intracellular scFv. Staining of EGFR expressing cells was observed (e.g., A431, MDA-MB-468 and CHO/EGFR) but no staining was seen for EGFR negative cells (MDA-MB-453 and CHO). Much of the scFv remained surface bound, with some intracellular staining observed (Figs. 5 and 6 for representative results with E12 scFv). To determine the ability of the scFv to deliver a drug to EGFR expressing cells, immunoliposomes were constructed by fusing E12 scFv to the surface of HPTS containing liposomes. Strong intracellular fluorescence was observed for EGFR expressing cells, with no fluorescence observed for EGFR negative cells.

4. Discussion

The first step in developing a targeted cancer therapeutic is generating a ligand that specifically binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. Antibodies have proved to be important targeting ligands for cell surface receptors, especially with recent engineering techniques to generate antibodies which are entirely human in sequence. Libraries of antibodies displayed on phage can rapidly generate panels of human antibodies to a target antigen without the need for immunization (Marks et al., 1991; Sheets et al., 1998). To generate antibodies which bind native cell surface receptors, we recently demonstrated that phage could be directly selected on tumor cell lines by recovering endocytosed phage from within the target cell (Poul et al., 2000). Compared to simply recovering phage from the cell surface, intracellular phage recovery increases specific enrichment of antigen binding antibodies more than 10- to 30-fold (Becerril et al., 1999). High enrichment ratios are essential for successful selection of antibodies on heterogeneous antigens such as the surface of cells. In our previous publication, more than 10 unique tumor specific antibodies were generated, two of which were determined to bind ErbB2 and the transferrin receptor (Poul et al., 2000).

For this work, we demonstrate that this approach can be used to generate human scFv antibodies to a known tumor antigen (EGFR). EGFR is a 170-kDa transmembrane glycoprotein overexpressed in a number of human cancers. Ligand binding induces receptor dimerization which results in autophosphorylation of the kinase domain (Odaka et al., 1997; Tzahar et al., 1997). Receptor internalization occurs following dimerization and is believed to be a mechanism of receptor signal downregulation. EGFR antibodies were generated both by selecting on an overexpressing cell line or by using a cell line transfected with the target gene. The transfected human EGFR has been shown to function normally in its foreign environment: stimulation with EGF leads to receptor phosphorylation and receptor internalization follows activation. Selection on the EGFR transfected cell line permits use of the untransfected parental cell line to deplete the library of phage binding irrelevant receptors, enhancing enrichment ratios. The two cell lines should differ only in the presence of the target receptor. The availability of the untransfected cell line also provides an ideal reagent for the screening and characterization of antigen specific clones following selection. The ability to select on a transfected cell also eliminates

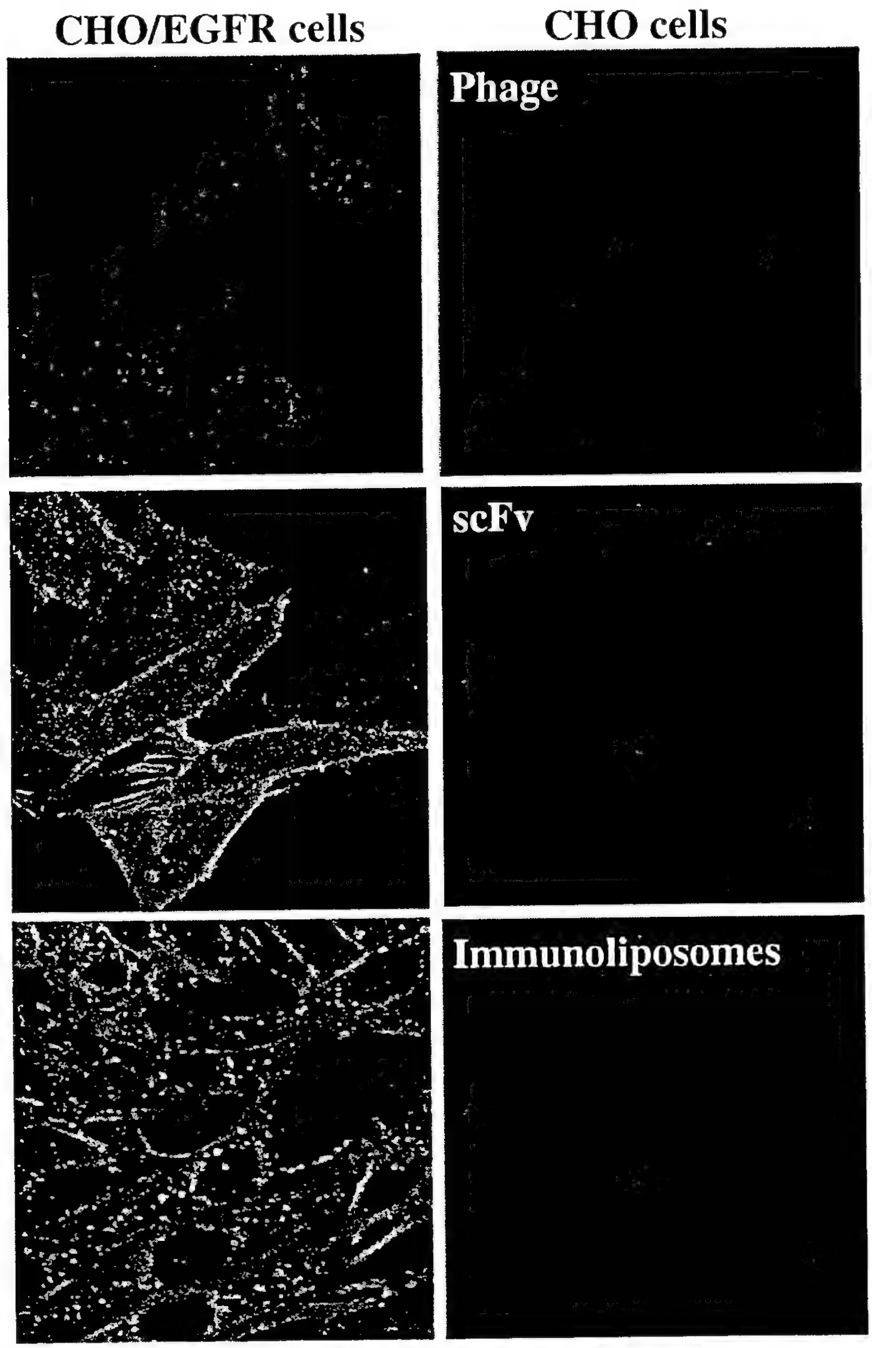

Fig. 5. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into CHO/EGFR and CHO cells. The E12 phage antibody was detected with α-M13-biotin followed by streptavidin–phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into CHO/EGFR and CHO cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv, and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

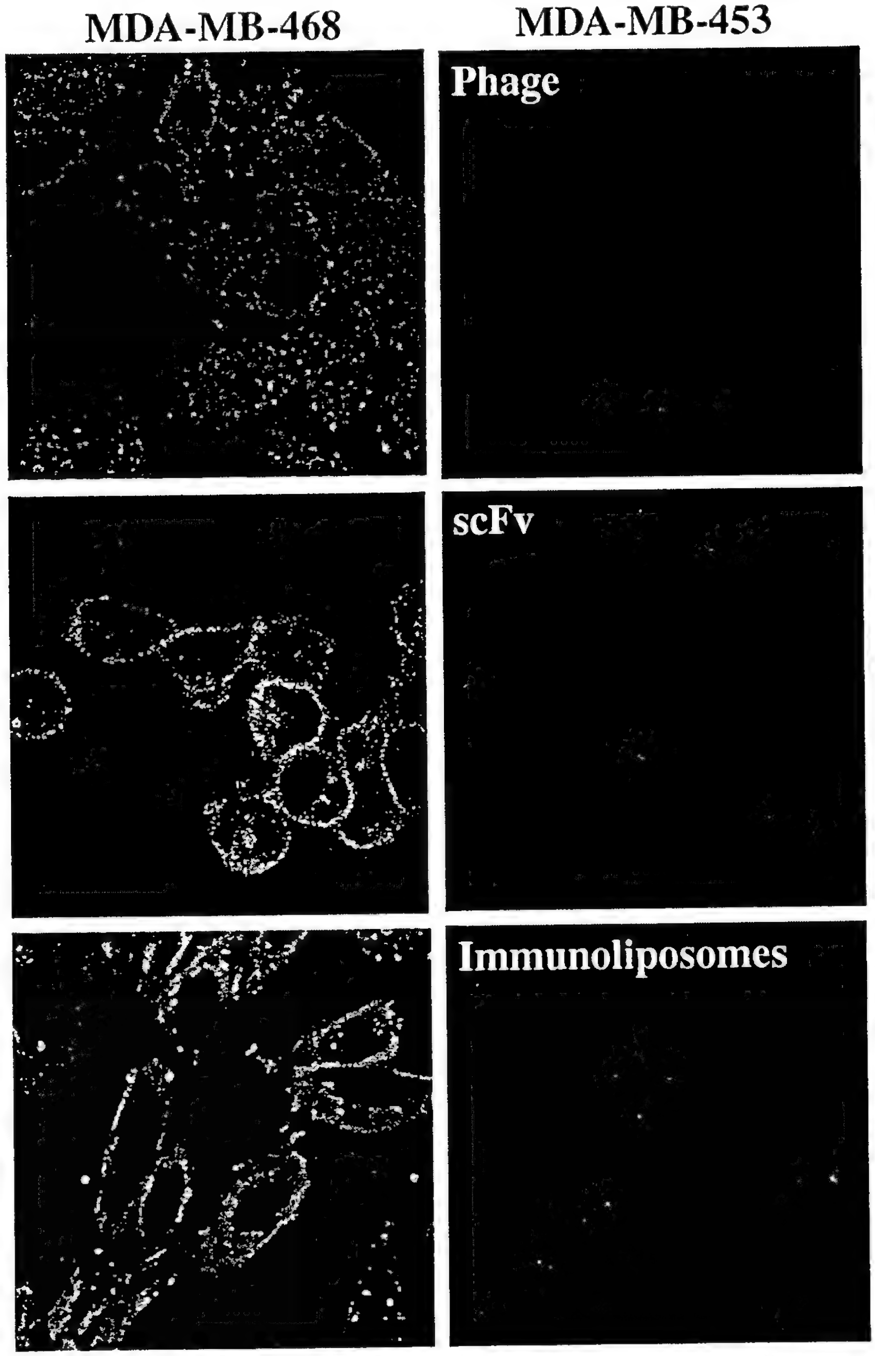

Fig. 6. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells. The E12 phage antibody was detected with α-M13-biotin followed by streptavidin–phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

the need to express and purify the target antigen in order to select antibodies. This could significantly speed development of antibodies to genes discovered as part of genomic sequences.

Selection on EGFR overexpressing A431 cells resulted in more efficient selection of EGFR antibodies than selection on CHO/EGFR cells (a higher percentage of antigen binding clones, although both selections yielded two unique antibodies). This occurred despite depletion of non-EGFR binding phage using the parental CHO cell line. In fact, the depletion was found to have been insufficient as FACS analysis showed that polyclonal phage bound both CHO/EGFR cells and CHO cells. The difference in efficiency between the two selections could potentially be attributed to a greater cell surface receptor density on A431 cells than on CHO/EGFR cells. Although not quantified, a Western blot of the cell lysates demonstrated a greater signal for A431 cells as compared to CHO/EGFR cells. Interestingly, one antibody was common to both selections (A431 or CHO/EGFR), whereas each of the remaining two antibodies were only selected on one of the cell types (A431 or CHO/EGFR). This result suggests that selection on multiple cell types may yield a greater number of antibodies.

The phage antibodies generated in this and previous work (Poul et al., 2000) were internalized by cells as determined by immunofluorescence and confocal microscopy. In both reports, the phage antibodies were selected from libraries where monomeric scFv were displayed as single copies in a phagemid system. In fact, all large non-immune libraries display monovalent antibody fragments (either scFv or Fab) as single copies using a phagemid vector. Since antibodies typically need to be bivalent to crosslink receptors and trigger endocytosis (Heldin, 1995; Yarden, 1990), successful selection of internalizing antibodies from phagemid libraries would require that: (1) the scFv formed spontaneous scFv dimers (diabodies) on the phage surface, as has been reported for some scFvs; (2) the monovalent scFv mimicked the natural receptor ligand leading to receptor aggregation and endocytosis or (3) increased phage display levels led to greater than one scFv per phage. In our previous work, the two scFvs studied extensively (anti-ErbB2 and anti-transferrin receptor) were stable scFv monomers in solution and were significantly endocytosed into cells as monomeric scFv. In the case of the transferrin receptor antibody, the scFv was a ligand mimetic and could compete with the natural ligand transferrin for binding to the receptor. In the case of the ErbB2 scFv, the mechanism by which it was endocytosed as a monomer is unknown. In the present work, the E12 scFv shows evidence of spontaneous dimerization (diabody formation) by gel filtration which could explain how it could crosslink receptors and trigger endocytosis. Interestingly, the purified scFv monomer (separated from dimer) shows significantly more surface membrane staining than intracellular staining (Figs. 5 and 6), especially compared to the multimeric immunoliposomes or to phage (which could be displaying dimeric scFv). In the case of the other two EGFR scFvs (which form stable monomers) the mechanism of endocytosis is unclear. We did not study whether the EGFR antibodies were ligand mimetics.

The approach described would be limited to those receptors capable of undergoing endocytosis. While this eliminates some useful cell surface targets, ligand binding and receptor internalization is a common mechanism for receptor and signaling regulation. Since most antibodies need to be bivalent to crosslink receptors and be efficiently endocytosed, one mechanism to increase the applicability of this selection methodology would be to construct bivalent diabody libraries in a phagemid vector or scFv libraries in a multivalent phage vector. This should open up the selection approach to more epitopes on more target antigens. Our model system results indicate that the most efficient selection format would be display on phage (Becerril et al., 1999), an approach which is presently under investigation.

The therapeutic utility of scFvs generated by this approach depends on the specific molecules to be targeted by the antibodies and the properties of the antibody. For many therapeutic approaches (immunotoxins, immunoliposomes, gene therapy) intracellular delivery of the toxic molecule is essential. Other approaches, for example bispecific antibodies or enzyme activated prodrugs, require that the antibody and effector molecule remain on the cell surface. Based on the present results (and our prior publication), the selection strategy described generates two types of scFv: those that are endocytosed in

their monomeric form (probably the majority of scFvs) and those that remain on the cell surface as monomers but are endocytosed when dimeric or multimeric. scFvs which are endocytosed as monomers could only be used for targeting effector molecules that are active intracellularly. scFvs which are primarily endocytosed as dimers could be used to leave effector molecules on the cell surface (when used as monomeric antibody fragments) or to deliver drugs intracellularly (when used as bivalent diabodies or IgG or when targeting multivalent nanoparticles).

In conclusion, we report the successful selection of EGFR antibodies from a phage library by selection for internalization into overexpressing cells or transfected cells. The scFvs are specific for EGFR expressing cells and can be used to target nanoparticles for intracellular drug delivery. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences.

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Phage vs phagemid libraries for generation of human monoclonal antibodies

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Abbreviations used. ELISA, enzyme linked immunosorbent assay; scFv, single chain Fv; PCR, polymerase chain reaction; TREM, triggering receptor expressed on myeloid cells; Kd, dissociation kinetics; TYE, tryptone yeast extract.

Abstract

Non-immune (naïve) phage antibody libraries have become an important source of antibodies for reagent, diagnostic, and therapeutic use. To date, reported naïve libraries have been constructed in phagemid vectors as fusions to pIII, yielding primarily single copy (monovalent) display of antibody fragments. For this work, we subcloned the single chain Fv (scFv) gene repertoire from a naïve phagemid antibody library into a true phage vector to create a multivalently displayed scFv phage library. Compared to monovalently displayed scFv, multivalent phage display resulted in improved efficiency of display as well as antibody selection. A greater number of antibodies were obtained and at earlier rounds of selection. Such increased efficiency allows the screening for binding antibodies after a single round of selection, greatly facilitating automation. Expression levels of antigen binding scFv were also higher than from the phagemid library. In contrast, the affinities of scFv from the phage library were lower than from the phagemid library. This could be overcome by utilizing the scFv in a multivalent format, by affinity maturation, or by converting the library to monovalent display after the first round of selection.

Introduction

Non-immune (naïve) phage antibody libraries have become an important source of human antibodies [1;2]. Large and diverse libraries can rapidly provide panels of single chain Fv (scFv) or Fab antibody fragments to virtually any antigen with affinities comparable to those of antibodies generated from hybridomas [3;4;5]. All reported naïve libraries have been constructed in phagemid vectors as fusions to pIII, partially due to the higher transformation efficiency of phagemid vectors compared to phage vectors. In phagemid systems, helper phage is provided *in trans* to supply the other phage genes and gene products for phage particle generation [1]. As a result, wild type pIII competes with antibody fragment-pIII fusion for incorporation into phage. The resulting phage population consists of phage bearing between 0 and 5 copies of antibody fragment per phage [1]. In fact, the majority of phage bear no antibody fragment, with the next most frequent phage bearing a single copy of antibody fragment. Such monovalent display has the potential advantage of allowing more efficient selection of higher affinity antibodies compared to mulivalent display as occurs with phage vectors. With multivalent display, the presence of multiple antibodies per phage permits avidity and a higher functional affinity when the antigen is multivalent, as occurs with solid phase immobilization of antigen.

Recently, we reported using a model system that display of an ErbB2 antibody fragment in the phage vector fd-tet resulted in more efficient cell surface enrichment on ErbB2 expressing cells than display of the same fragment in a phagemid system [6]. Moreover, multivalent phage display led to ErbB2 receptor crosslinking and receptor mediated endocytosis of the phage antibody. Such endocytosis was significantly more efficient than with phagemid display [6]. To more efficiently select phage antibodies which

trigger receptor mediated endocytosis [7;8], we constructed a naïve phage antibody library in the phage vector fd-tet [9;10]. We previously reported that display of scFv on phage resulted in more efficient selection of antibodies using antigen blotted onto nitrocellulose then with phagemid libraries [9] and also reported the successful selection of fetal erythrocyte antibodies by direct selection of a phage antibody library on fetal erythrocytes [10]. These successes led us to hypothesize that phage libraries might result in more efficient selection of antibodies on less complex antigens, such as purified proteins or peptides, compared to phagemid libraries. To test this hypothesis, we selected naïve phage and phagemid antibody libraries on 2 proteins and 1 phosphopeptide using the most commonly employed method of antigen immobilization, coating of immunotubes. We compared the efficiency of antibody display, efficiency of antibody selection, diversity of antibodies generated and the binding constants and expression levels of the selected antibodies. The results have implications for library design, selection methodology, and ease of selection automation.

Results

Library construction and characterization

A 7.0 x 10^9 member non-immune human scFv phagemid antibody library has been previously constructed and characterized [4]. To create a non-immune human scFv phage antibody library, the scFv gene repertoire was excised from the phagemid library and subcloned into the phage vector fd-tet-Sfi/Not [11] (Figure 1). After ligation and transformation of *Escherichia. coli*, a library of 5 x 10^8 tetracycline resistant colonies was obtained [9;10]. PCR screening indicated that 20/20 clones contained a scFv sized insert, giving a library size of 5 x 10^8. To determine the relative efficiencies of scFv display on the phage pIII, polyclonal phage was prepared from the phage and phagemid libraries.

Phage prepared from the two libraries was subjected to SDS-PAGE followed by Western blotting, with detection using anti-pIII antibody. The amount of scFv-pIII fusion protein was significantly greater in the phage library compared to the phagemid library, as was the ratio of scFv-pIII fusion compared to wild-type pIII (Figure 2A). Similar analysis was performed on ten naïve clones selected at random from the two libraries. All ten clones from the phage library had detectable scFv-pIII fusion protein compared to only 3 of 10 clones from the phagemid library (Figure 2B). For clones with detectable fusion protein, the ratio of fusion protein to wild type pIII was greater for the clones from the phage library.

Selection of antigen binding phage antibodies

Phage and phagemid libraries were subjected to three rounds of selection on three different antigens immobilized on immunotubes: two protein antigens, PcrV and TREM 2a, and one phosphopeptide, ErbB2 Y1023 (DLVDAEEYLVPQQGF). After each round of selection, the frequency of antigen binding phage antibodies was higher from the phage antibody library compared to the phagemid library for all three antigens (Table 1). For two of the antigens, 5% to 6% of clones bound antigen after a single round of selection using the phage library. In contrast, only a single binder was detected after the first round of selection using the phagemid library. After two rounds of selection, 35%-78% of clones bound antigen using the phage library (Table 1). In contrast, 2% to 29% of clones bound antigen after the second round of selection using the phagemid library. To determine the impact of selection on the diversity of the selected antibodies, the scFv gene from antigen binding clones was subjected to PCR fingerprinting [12]. All antigen binding clones from the second and third rounds of selection for both libraries on each antigen were fingerprinted (Table 2, and Figure 3). After the second round of selection, a

much larger number of unique antibodies were identified from the phage library (8 to 23) compared to the phagemid library (2 to 9) for all antigens. Across the three antigens there was between 1.66 - 5.75 times as many unique clones from the fd-tet library than the phagemid library. After a third round of selection, the number of unique antibodies decreased for both types of libraries, with the phage library still showing a greater number of unique antibodies compared to the phagemid library.

Further characterization of selected clones

To determine the impact of the display system on the equilibrium binding constants of the antibodies, the scFv genes from PcrV binding phage antibodies were sublconed into the vector pUC119 Sfi-NotmycHis, resulting in the fusion of a hexa-histidine tag at the C-terminus of the scFv [13]. ScFv was expressed, harvested from the bacterial periplasm, and purified by immobilized metal affinity chromatography (IMAC) followed by gel filtration to remove any aggregated or dimeric scFv. ScFv from three phagemid and eight phage clones were purified, with the expression yields greater for the phage clones compared to the phagemid (Table 3). Association and dissociation rate constants were measured using surface plasmon resonance in a BIAcore and used to calculate the equilibrium dissociation constant (K_d). Two of the three K_d were below 50 nM from the phagemid library, with the lowest affinity scFv having a K_d of 140 nM (Table 3). In contrast, all K_ds of scFv from the phage antibody library were higher (lower affinity) than scFv from the phagemid library, ranging from 148 to 1160 nM (Table 3).

Discussion

For this work, we compared human scFv phage antibody libraries constructed in phage and phagemid vectors. The library in a phage vector yielded a greater number of scFv antibodies per antigen than the phagemid library (15.0 vs 5.7). The number of

antibodies per antigen is also greater than reported for 10 other phagemid antibody libraries (Table 4). This is likely due to the fact that multiple copies of antibody fragment allow multivalent binding to antigen [14], thus allowing selection of scFv with monovalent binding constants that are either too low to be selected or are inefficiently selected when present in a single copy on phage. For the phage library, the frequency of antigen binding antibodies was also greater at each round of selection. In fact, a relatively high frequency of antigen binding antibodies were present after a single round of selection from the phage library (5-6%). Such improved selection efficiency likely results from the increased efficiency of antibody fragment display, as well as an increase in the functional binding constant due to an avidity effect resulting from display of multiple copies of antibody fragment [14]. The net result of increased selection efficiency is that it is possible to screen for binding antibodies after a single round of selection. Screening after a single round of selection greatly simplifies automation of the selection process, since it is not necessary to amplify phage for subsequent rounds of selection by culturing.

The mechanism of improved antibody fragment display in phage vectors compared to phagemid vectors is unclear. In the phage vector, expression of the pIII-scFv fusion is driven by the natural phage promoter and no helper phage is required for phage particle generation [15; 16; 17]. A powerful transcriptional terminator is present after pVIII prior to the pIII gene, and the pIII gene initiation codon is a valine [15; 16]. All of the above features likely result in minimal, yet perfectly regulated pIII expression [18]. In phagemid vectors, pIII-scFv expression is typically driven by the lac promoter [17; 19]. Expression of scFv-pIII fusion protein results either from leaky expression after the exhaustion of the inhibitor glucose from the media, or by addition of small amounts of IPTG if the lac repressor is included in the phagemid vector. Since both pIII and scFv protein is toxic to *E. coli*, increased expression, as may occur using artificial promoters,

may actually lead to less, rather than more, fusion protein [18; 20; 21]. In addition, phagemid systems require the addition of helper phage for phage particle generation. Wild-type pIII from the helper can compete with pIII-scFv fusion protein for incorporation into the phage particle [1]. Furthermore, infection by helper phage is hindered or blocked in the presence of pIII expression in *E. coli* [22]. Thus leaky expression of pIII-scFv fusion may prevent subsequent infection with helper phage. Since different scFv genes and gene product sequences have differential toxic effects on *E. coli* [20], different library members may differ significantly in the rate at which they are rescued and packaged by helper phage infection. This effect may explain why scFv from the phage library had higher expression levels than scFv from the phagemid library. ScFv with higher levels of expression may result in higher levels of pIII-scFv fusion protein in *E. coli* and inhibition of infection with helper phage. Such scFv would not be displayed and hence would not be selected.

In contrast to the improved efficiency of selection, the monovalent affinities of the selected scFv antibodies were lower from the phage library compared to the phagemid library. There are two possible explanations. First, multicopy display in phage vectors results in an avidity effect allowing the selection of antibodies with a lower monovalent binding constant than are selectable from the monovalent display phagemid system [6; 14]. In a repertoire of given size, the frequency of lower affinity binders will be greater than the frequency of higher affinity binders [23]. Thus lowering of the selection affinity threshold by itself biases for an increased frequency of lower affinity antibodies. Moreover, the greatest increment in affinity when antibodies are multimerized occurs with the lowest affinity antibodies [24]. Thus multivalent display minimizes the differences in monovalent binding constants, thus reducing the selection for higher affinity binders that normally occurs with monovalent display. It might be possible to overcome this

avidity effect by selecting on soluble antigen in solution [25]. The second explanation for selection of lower affinity antibodies from the phage library is that the library size was 14 times smaller than the phagemid library. However, since all members of the phage library showed detectable scFv-pIII display compared to only 30% of the phagemid library, it is likely that functional library size differed only by 4 fold. Larger libraries yield antibodies of higher affinity [23; 26] (Table 4). While we could have corrected for the difference in library size by using only a portion of the phagemid library, the current analysis more accurately reflects the possible library sizes that can be reasonably generated from the two systems; phage transfection efficiencies are significantly lower than phagemid.

In conclusion, we have shown that phage libraries result in greater antibody display levels and more efficient selection on antigen. A greater number of antibodies are also generated, but are of lower affinity than from phagemid libraries. Phage libraries may be more useful than phagemid libraries for automated selections, especially if secondary screens for affinity are available, or when antibodies with rare biologic properties are desired. If the affinities of the selected antibodies are inadequate, then in vitro affinity maturation can be performed. Alternatively, the phage library could be converted to primarily monovalent display after a single round of selection (either by subcloning into a phagemid vector or by treating the phage with trypsin). Phage libraries are also probably more useful for cell selections, especially for triggering of receptor mediated endocytosis [6; 10]. Phage libraries will also be useful for antibody generation for applications where the antibody will be used in multiple copies and the monovalent binding constant is not important. In contrast, phagemid libraries appear more useful when the highest affinity antibodies are desired with minimal secondary screening.

Methods

Library construction and characterization

For the non-immune phagemid library, a previously reported human scFv antibody library containing 6.7×10^9 members was utilized [4]. For the non-immune phage library, scFv genes were excised as Sfi1-Not1 fragments from DNA prepared from the 6.7×10^9 member phagemid library and gel purified. The scFv gene repertoire was ligated into the phage vector fd-SfiI/NotI [7] (provided by Dr. Andrew Griffiths, MRC, Cambridge). Ligation mixtures were used to transform *E. coli* TG1 and the transformation mixture plated on TYE plates containing 15 µg/ml tetracycline (TYE-Tet plates). Library size was calculated by counting the number of tetracycline resistant colonies. Library quality was verified by determining the percentage of clones with an insert the appropriate size for an scFv gene by colony PCR screening using the primers fdseq [27] and fd2, [10]. Library diversity was confirmed by BstN1 fingerprinting the amplified scFv genes [12] as described in reference 10. The library was stored in 2 x TY containing 15 µg/ml tetracycline and 15% glycerol at -80°C.

For the phagemid library, phagemid particles were prepared by rescue withVCS-M13 helper phage (Stratagene) as previously described [27]. For the phage library, phage particles were prepared by inoculation of 1L of 2 x TY containing 15 µg/ml tetracycline (2 x TY-Tet) with an aliquot of library glycerol stock and the culture grown overnight at 30°C with shaking at 250 rpm. Phage were harvested by centrifugation, concentrated by PEG precipitation [27] and purified by CsCl gradient centrifugation as described. Phage concentration was determined by titering on *E. coli* TG1.

The extent of scFv-pIII fusion was determined for both polyclonal phage prepared from the libraries and for randomly picked individual clones by Western blotting. For Western blot, 3×10^{12} phage were boiled in denaturing SDS buffer, subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% milk/PBS for 60 min at RT. Membranes were incubated with a 1:3000 dilution of pIII antibody (Mo Bi Tec) in 5% milk/PBS for 60 min at RT. Membranes were washed in PBS/0.05% Tween and incubated with a 1:1000 dilution of anti-mouse/HRP (Santa Cruz) for 30 min at RT. Membranes were further washed in PBS/0.05% Tween and the HRP conjugate detected with ECL detection reagent (Amersham Pharmacia).

Selection of Phagemid and Phage Antibodies.

Libraries were selected using 75 mm x 12 mm immunotubes (Nunc; Maxisorb) coated overnight at 4°C with 2 ml of 50 ug/ml PcrV [28], 50 ug/ml TREM 2a [29], or the tyrosine phosphorylated peptide P-Y1023 (DLVDAEEYLVPQQGF) taken from the cytoplasmic domain of human ErbB2 [30]. For phosphopeptide selections, peptide was first conjugated to maleimide activated BSA (Pierce) as described by the manufacturer. Tubes were coated with 10 μg/ml of BSA-peptide conjugate. Tubes were blocked with 2% skimmed milk powder in PBS for 1 hour at room temperature, and then the selection, washing and elution procedures were performed as previously described [27] using phage at a concentration of 5.0×10^{12} TU/ml. 500 μl of the eluted phage were used to infect 10 ml log phase growing *E. coli* TG1, which were plated on TYE plates containing 100 μg/ml ampicillin and 1% glucose (TYE-AMP-Glu plates) (phagemid library) or on TYE-Tet plates. Phage or phagemid particles were prepared and concentrated by PEG precipitation and used for the next selection round. The phage preparation-selection-plating cycle was repeated for three rounds.

Phage ELISA

Antigen binding phage antibodies were identified by phage ELISA. For ELISA, individual colonies were picked into 96 well microtiter plates containing 2 x TY-AMP-GLU (phagemid library) or 2 x TY-Tet (phage library). For phagemid libraries, phage particles were rescued by the addition of VCS-M13 helper phage as previously described [27]. Bacteria were grown overnight at 30°C, the bacteria pelleted, and supernatant containing phage particles used for ELISA. For ELISA, microtiter plates (Falcon 353912, Becton Dickinson) were coated overnight at 4°C with 50 µl/well of 10 µg/ml of PcrV, 10 µg/ml of Trem 2a, or 10 µg/ml of BSA-peptide conjugate. The next day, wells were blocked for 2 hours at RT with 2% skimmed milk powder in phosphate buffered saline (PBS). Phage binding was detected with anti-M13 antibody (Amersham Pharmacia) diluted 1:3000 in PBS followed by ABTS as previously described [27]. The number of unique phage antibodies was estimated by PCR fingerprinting of the scFv genes with the restriction enzyme *Bst*NI as previously described [27].

ScFv Purification and Affinity Measurements

For purification, scFv genes were subcloned into the expression vector pUC119 Sfi-NotmycHis, resulting in the fusion of a hexa-histidine tag at the C-terminus of the scFv [13]. ScFv was expressed and purified by immobilized metal affinity chromatography followed by gel filtration on a Sephadex 75 column to remove aggregated or dimeric scFv as previously described [25]. The concentration of purified monomeric scFv determined spectrophotometrically, assuming an A_{280} nm of 1.0 correlates to an scFv concentration of 0.7 mg/ml.

Association (k_{on}) and dissociation (k_{off}) rate constants were measured using surface plasmon resonance in a BIAcore and used to calculate the equilibrium

dissociation constant. In a BIAcore flow cell, approximately 600 resonance units (RU) of PcrV (15 μg/ml in 10 mM sodium acetate, pH 4.5) were coupled to a CM5 sensor chip using NHS-EDC chemistry [17]. This amount of coupled PcrV resulted in scFv RU_{max} of 100-175 RU. The surface was regenerated after binding of scFv using 4 M $MgCl_2$. The K_d of scFv was calculated from the association (k_{on}) and dissociation (k_{off}) rate constants determined in the BIAcore ($K_d = k_{off}/k_{on}$). Association was measured under continuous flow of 5 μl/min using a concentration range of scFv from 50 to 1000 nM. Association rate constant (k_{on}) was determined from a plot of (ln (dR/dt))/t vs. concentration [31]. Dissociation rate constant (k_{off}) was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed using a flow rate of 30 μl/min to prevent rebinding.

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Table 1. Frequency of antigen binding phage antibodies as a function of selection round. Phage and phagemid libraries were selected on antigen and the frequency of binding phage antibodies determined by ELISA. 96 clones from each round of selection were screened.

Antigen	Round1 Phage	Round 2 Phage	Round 3 Phage	Round 1 Phagemid	Round 2 Phagemid	Round 3 Phagemid
ErbB2 Y1023	5.4%	39.1%	93.6%	1.1%	6.5%	93.6%
PcrV	6.5%	78.3%	97.9%	0%	28.7%	95.7%
TREM 2a	0%	34.8%	100%	0%	2.2%	47.8%

Table 2. Effect of phage antibody library type and selection round on the number of unique antibodies identified. All antigen binding clones from the 96 clones screened from each round of selection were subjected to PCR fingerprinting to determine the number of unique antibodies present. Numbers represent the number of unique antibodies identified/the number of antigen binding clones screened.

Antigen	Phage library		Phagemid library	
	Round 2	Round 3	Round 2	Round 3
ErbB2 Y1023	23/35	11/88	4/6	7/88
PcrV	14/72	8/92	4/24	9/90
TREM 2a	8/32	9/94	2/2	4/44

Table 3. Affinities, binding kinetics and expression levels for anti-PcrV phagemid and phage clones. Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFv were measured by using surface plasmon resonance (BIAcore) and Kd was calculated as (k_{off}/k_{on}).

Phagemid clone	**Kd (x 10^{-9} M)**	**k_{on}(x 10^5 $M^{-1}s^{-1}$)**	**k_{off} (x$10^{-3}s^{-1}$)**	**Expression level (mg/ml)**
PcrV SA2	32	6.05	0.0197	0.033
PcrV SA7	37	5.68	0.0231	0.46
PcrV SE1	141	2.02	0.0285	0.28
Phage clone				
PcrV FD6	148	2.86	0.0426	0.18
PcrV FC11	227	2.63	0.0615	1.10
PcrV FD3	400	1.06	0.0464	1.14
PcrV FA5	687	5.38	0.37	1.10
PcrV FF4	791	.812	0.0643	1.10
PcrV FG2	819	.183	0.015	0.50
PcrV FE2	992	.388	0.0385	0.50
PcrV FA11	1160	.541	0.0628	0.53

Table 4. Comparison of protein binding antibodies selected from non-immune phage-display antibody libraries. All libraries were constructed in phagemid vectors except for this work.

Library	Library size and type	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens (x 10^{-9} M)
Marks et al. (ref. 27)	3.0×10^7 (scFv, N)	2	2.5	1	100-2000
Nissim et al. (ref. 32)	1.0×10^8 (scFv, SS)	15	2.6	ND	ND
DeKruif et al. (ref. 33)	3.6×10^8 (scFv, SS)	12	1.9	3	100-2,500
Griffiths et al. (ref. 11)	6.5×10^{10} (Fab, SS)	30	4.8	3	7.0-58
Vaughan et al. (ref. 3)	1.4×10^{10} (scFv, N)	3	7.0	3	4.2-8.0
Sheets et al. (ref. 4)	6.7×10^9 (scFv, N)	14	8.7	8	0.22-71.5
Little et al. (ref. 34)	4.0×10^9 (scFv, N)	2	3.5	ND	ND
Sblattero & Bradbury (ref. 35)	2.0×10^9 (scFv, N)	7	6.14	4	15.6-59.8
Soderlind et al. (ref. 36)	3.0×10^{11} (scFv, N)	6	3.67	2	3.1-7.6
de Haard et al. (ref. 5)	3.7×10^{10} (Fab, N)	6	14	3	2.71-38.8
O'Connell et al. (this work)	5×10^8 (scFv, N)	3	15.3	11	148-1160

Figure legends

Figure 1. Schematic representation of the sub-cloning strategy employed to transfer the scFv gene repertoire of the phagemid library from the phagemid vector pHEN1 into the phage vector fd-SfiI/NotI. A 1.3 Kb stuffer fragment was first sub-cloned into the phage vector fd-tet using the restriction sites *SfiI/NotI* to facilitate the subsequent sub-cloning of the scFv repertoire into this vector. These restriction sites were used to excise the scFv gene repertoire from pHEN1. The *SfiI* site is located within the pelB gene sequence and the NotI restriction site abuts sequence coding for 3 alanine residues 5' to a c-myc epitope tag and an amber stop codon before the gene III sequence. Sub-cloning of the scFv repertoire into fd-tet places it in frame with a hybrid gene III-pelB leader sequence and abutting the alanine coding sequence immediately 5' of the gene III sequence.

Figure 2. Anti-pIII western blot of total library phage (A) and of randomly picked unselected clones (B). Phage particles were prepared from phage or phagemid libraries and electrophoresed on a 10% polyacrylamide gel. Helper phage was used as a control to indicate the location of wild-type pIII. PIII and scFv-pIII fusions were detected with a pIII antibody. Polyclonal or monoclonal phage antibodies demonstrated significantly more scFv-pIII fusion than polyclonal or monoclonal phagemid antibodies.

Figure 3. Genotype of anti-PcrV scFv from the second round of the phagemid and phage library selections. The BstNI digest of the scFv genes identifies 4 unique phagemid clones with 14 unique phage clones.

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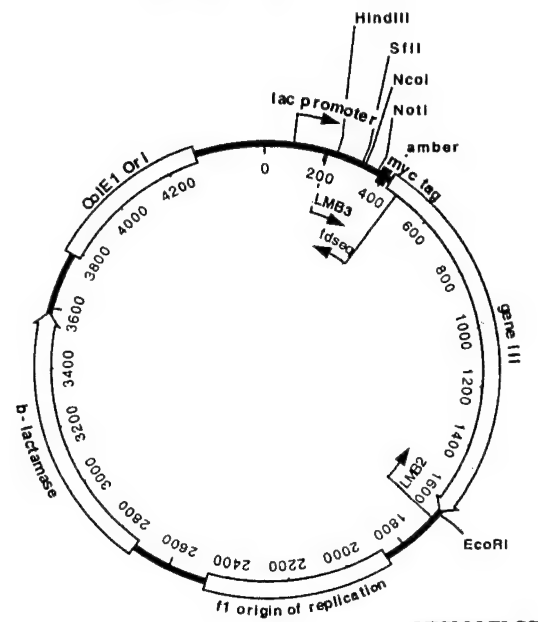


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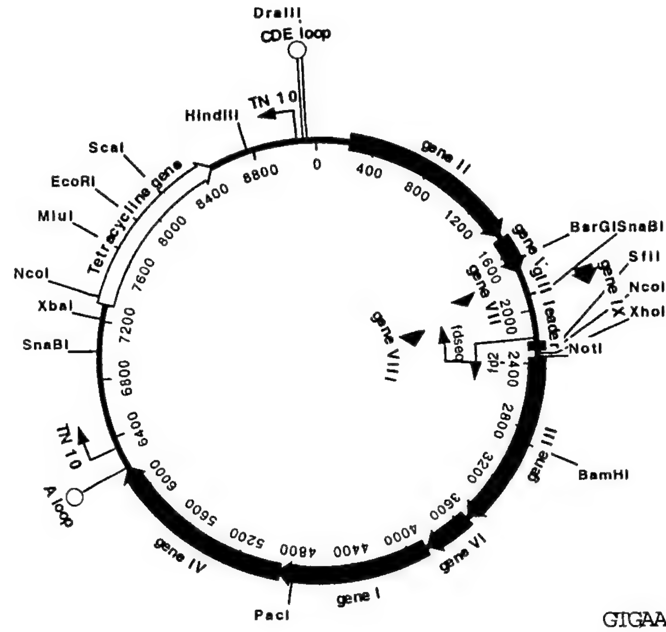


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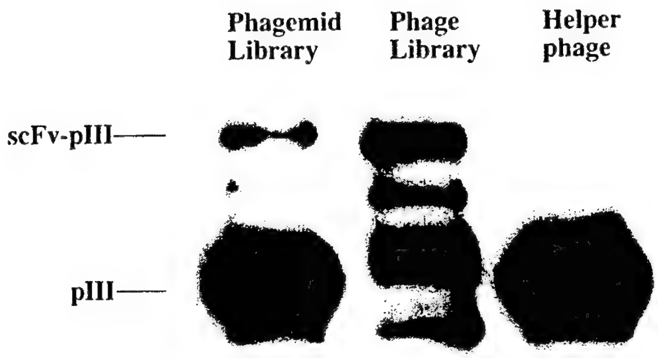
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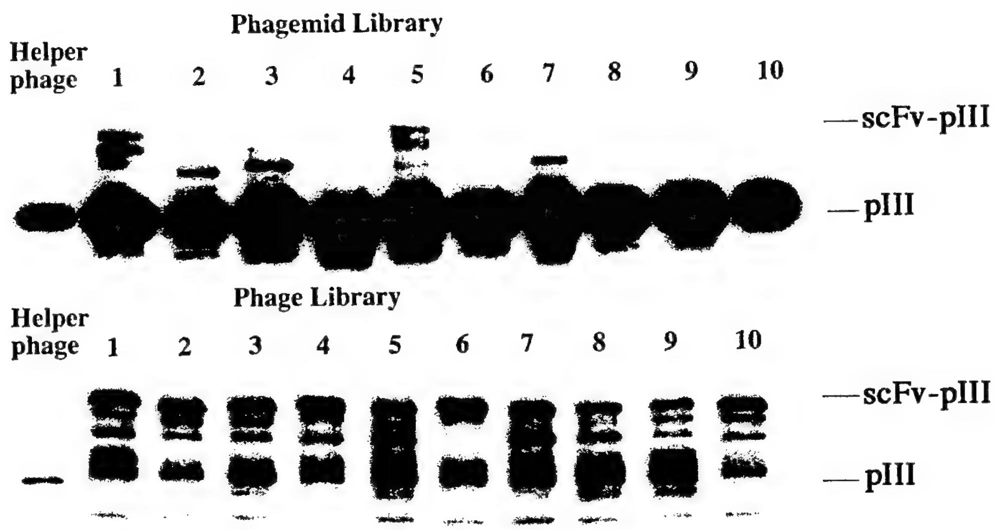
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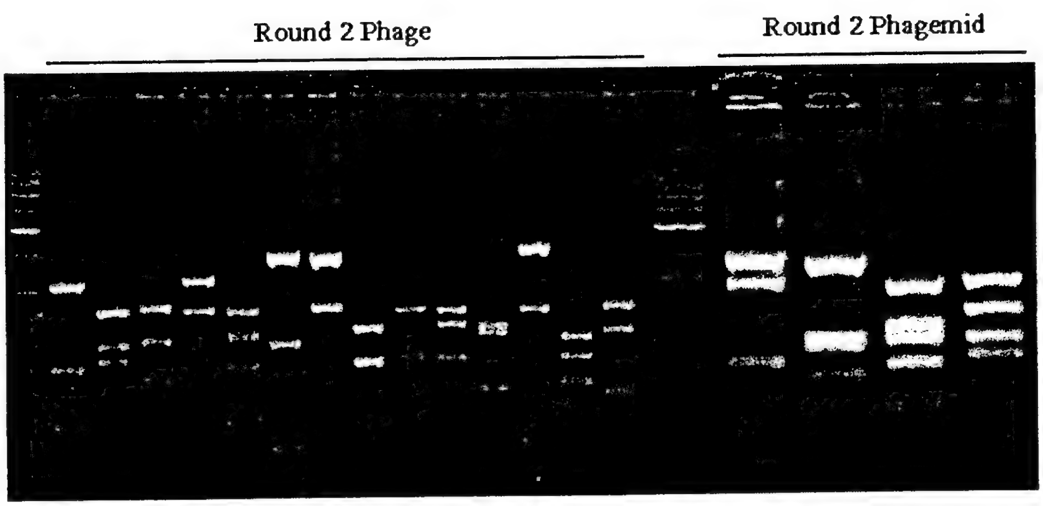
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Antibodies to human fetal erythroid cells from a nonimmune phage antibody library

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Contributed by Yuet W. Kan, December 29, 2000

The ability to isolate fetal nucleated red blood cells (NRBCs) from the maternal circulation makes possible prenatal genetic analysis without the need for diagnostic procedures that are invasive for the fetus. Such isolation requires antibodies specific to fetal NRBCs. To generate a panel of antibodies to antigens present on fetal NRBCs, a new type of nonimmune phage antibody library was generated in which multiple copies of antibody fragments are displayed on each phage. Antibody fragments specific for fetal NRBCs were isolated by extensive predepletion of the phage library on adult RBCs and white blood cells (WBCs) followed by positive selection and amplification on fetal liver erythroid cells. After two rounds of selection, 44% of the antibodies analyzed bound fetal NRBCs, with two-thirds of these showing no binding of WBCs. DNA fingerprint analysis revealed the presence of at least 16 unique antibodies. Antibody specificity was confirmed by flow cytometry, immunohistochemistry, and immunofluorescence of total fetal liver and adult RBCs and WBCs. Antibody profiling suggested the generation of antibodies to previously unknown fetal RBC antigens. We conclude that multivalent display of antibodies on phage leads to efficient selection of panels of specific antibodies to cell surface antigens. The antibodies generated to fetal RBC antigens may have clinical utility for isolating fetal NRBCs from maternal circulation for noninvasive prenatal genetic diagnosis. Some of the antibodies may also have possible therapeutic utility for erythroleukemia.

antibody phage display | monoclonal antibody | single chain Fv | fetal erythroid antibodies

It has long been known that fetal red blood cells (RBCs) routinely leak into the maternal circulation during normal pregnancy (1, 2). More recently, it has been established that a very small number of fetal nucleated RBCs (NRBCs) are also routinely present in the maternal circulation (3, 4). These cells are considered the ideal target for noninvasive DNA prenatal diagnosis, but presently they cannot be readily isolated from the maternal circulation in high enough numbers and purity for routine clinical use. Because the isolation methods for purifying fetal NRBCs from maternal circulation rely on antibody-based separation and detection techniques, progress in this area has been hampered by the relative lack of antibodies to unique fetal erythroid antigenic determinants (5). Well characterized antigens expressed on fetal erythroid cells but not adult RBCs, such as CD71 and CD36, are also expressed on a number of adult white blood cells (WBCs) resulting in contamination by many WBCs in purification techniques relying on these antibodies.

Fetal erythroid lineage antigens classically have been identified by massive screening of mAbs produced by conventional murine hybridoma technology using mice immunized with human fetal NRBCs. The majority of antibodies generated by this method are nonspecific and react with irrelevant epitopes present on all human cells. Conventional murine hybridoma technology also tends to produce antibodies only to immunogenic antigens, because it relies on natural immune response in an animal. Thus, antibodies to antigens that are strongly evolutionarily conserved tend not to be produced by this technology.

To overcome these limitations, we applied antibody phage display technology to isolate new fetal erythroid lineage specific antibodies. In antibody phage display, large nonimmune libraries are created and display single-chain variable antibody fragments (scFv) on the surface of filamentous bacteriophage virions (refs. 6 and 7; reviewed in ref. 8). The gene for the displayed antibody is carried in the phage genome, thus linking genotype with phenotype. Antigen specific antibodies are selected from the library by a variety of different affinity chromatography techniques. Because this approach does not depend on a natural immune response and uses entirely *in vitro* selection techniques, antibodies can be isolated to any antigens, including nonimmunogenic and conserved antigens (9–11). Antibodies to cell surface antigens can be directly isolated from phage antibody libraries by panning on cells, including blood cells (12, 13). In fact, RBCs were the first cell type used to demonstrate the feasibility of cell surface selection by antibody phage display (12). Such cell selections, however, have not proven generally successful for generation of panels of cell-type specific antibodies. Here we describe the generation of a new type of nonimmune phage antibody library in which multiple copies of antibody fragments are displayed on each phage and report its successful application to generate a panel of antibodies to unique fetal erythroid cell surface markers.

Methods

Blood Cell Preparations. Buffy coats containing peripheral blood leukocytes were obtained from the Irwin Memorial Blood Bank (San Francisco). Fetal livers of gestational ages ranging from 14–24 weeks were obtained from San Francisco General Hospital with the approval of the University of California, San Francisco Committee for the Protection of Human Subjects. For phage antibody selection and immunocytochemistry, fetal erythroid cells were isolated from the human fetal liver by straining through 70 μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ) to remove fetal hepatocytes and clumped cells, followed by panning on polystyrene plates coated with anti-glycophorin A (GPA) antibodies (Beckman Coulter, Westbrook, ME) at 10 μg/ml in 0.5 M Tris·HCl (pH 9.5) as follows: fetal cells were resuspended in 3 ml of PBS supplemented with 5% FCS at a concentration of 10^7 cells/ml and allowed to attach for 2 h at 4°C. Cells that did not attach were removed by washing four times with PBS/1% FCS.

For flow cytometry, light-density fetal liver cells, containing a high proportion of immature erythroid progenitors, were isolated by first homogenizing the liver through a wire mesh and washing the cells in PBS containing 0.5% fraction-V ethanol-

Abbreviations: scFv, single-chain variable antibody fragment; RBC, red blood cell, WBC, white blood cell; NRBC, nucleated RBC; GPA, glycophorin A; PEG, polyethylene glycol; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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extracted BSA (Boehringer Mannheim), and 50 μg/ml gentamicin (GIBCO/BRL). The fetal liver cells were next layered on a 1.077 g/ml solution of Nycoprep (GIBCO/BRL) and centrifuged at 1,000 × g for 25 min at room temperature. The cells were washed and resuspended in PBS/0.5% BSA for phenotypic analysis. Light-density fetal liver cells depleted of GPA^+ cells were prepared by immunomagnetic bead depletion as described (14).

Phage Display Library Construction. To generate phage displaying multiple copies of antibody fragment, an scFv phage antibody library was constructed in fd phage. The fd phage display library (B.B., Dave O'Connell, and J.D.M., unpublished work) was derived from a 7×10^9 member phagemid library (11) by subcloning the *Sfi*I/*Not*I scFv insert from pHEN1 into fd-*Sfi*1/*Not*I (15), (provided by Andrew Griffiths, Medical Research Council, Cambridge, U.K.). Ligation mixtures were used to transform *Escherichia coli* TG1 and the transformation mixture plated on TYE plates containing 15 μg/ml tetracycline. Library size was calculated by counting the number of tetracycline-resistant colonies. Library quality was verified by determining the percentage of clones with inserts of appropriate size for an scFv gene, performed by colony PCR screening using the primers fdseq (7) and fd2, 5′-TTTTTGGAGATTTTCAAC-3′. Library diversity was confirmed by *Bst*N1 fingerprinting the amplified scFv genes (16) as described in ref. 7. The library was stored in 2× TY containing 15 μg/ml tetracycline and 15% glycerol at −80°C.

Phage Library Preparation and Selection. Phage were prepared by inoculation in 1 liter of 2× TY containing 15 μg/ml tetracycline with an aliquot of library glycerol stock, and the culture was grown overnight at 30°C with shaking at 250 rpm. Phage were harvested by centrifugation, concentrated by precipitation with polyethylene glycol (PEG) (7), and purified by CsCl gradient centrifugation as described (17). Phage concentration was determined by titering on *E. coli* TG1.

Before selection, the phage library was extensively depleted against a mixture of adult RBCs and WBCs. A total of 10^{12} phage particles were incubated with 10^9 adult RBCs and 10^8 adult WBCs in PBS/1% BSA in a total volume of 1 ml for 15 min at room temperature with rotation. After incubation, phage binding adult RBCs and WBCs were removed by centrifugation and collection of the supernatant. The supernatant was used to resuspend fresh adult RBCs and WBCs. This procedure was repeated six times each with adult RBCs and WBCs The supernatant was further depleted of phage binding adult RBCs by incubation for 60 min at 4°C with adult RBCs attached to 15-cm polystyrene plates coated with 10 μg/ml anti-GPA antibodies. This supernatant was further depleted of phage binding WBCs by incubation for 60 min at 4°C with adult human WBCs attached to 15-cm polystyrene plates coated with 10 μg/ml anti-CD45 and anti-CD13 (Caltag, Burlingame, CA).

After the depletion steps, supernatant containing phage was incubated at 4°C for 60 min with fetal NRBCs attached to 10-cm polystyrene plates coated with 10 μg/ml anti-GPA antibodies. Plates were washed 10 times with 10 ml of ice-cold PBS/0.5% BSA. After washing, the fetal RBCs were scraped off the plates, washed twice with PBS/0.5% BSA and collected by centrifugation. After washing, fetal RBCs were lysed with 1 ml of 100 mM Triethlyamine (Sigma). The lysate was neutralized with 0.5 ml of 1 M Tris·HCl (pH 6.8) and then used to infect 10 ml of exponentially growing *E. coli* TG1 as described (7). *E. coli* was grown at 37°C for 1 h with shaking at 250 rpm after which time the culture was plated on TYE plates containing 15 μg/ml tetracycline. After overnight growth, colonies were scraped from the plates and used to generate phage for a second round of selection using depletion and positive selection steps as described above. The number of unique scFv was estimated by PCR fingerprinting of the scFv genes with the restriction enzyme *Bst*NI.

Flow Cytometry. For flow cytometry, phage was prepared from individual colonies. Phage was prepared by inoculation of 500 ml of 2× TY containing 15 μg/ml tetracycline with individual clones and grown overnight at 30°C. Phage were concentrated by PEG precipitation and resuspended in 2 ml of PBS/1% BSA. FACS analysis was performed on a FACScan or FACSCalibur flow cytometer (Becton Dickinson Immunchemistry Systems).

Blood cells were prepared as described above, washed with PBS/1% BSA and then incubated with phage. Approximately $1\text{–}5 \times 10^5$ cells and 10^{12} phage were incubated on ice for 1 h in 100 μl of PBS/1% BSA. After washing twice, cells were resuspended in 100 μl of biotinylated polyclonal anti-M13 (5 Prime → 3 Prime) diluted 1:2,000 in PBS/1% BSA and incubated on ice for 30 min. After being washed twice, cells were resuspended in either streptavidin-R-phycoerythrin (PE) conjugate (Molecular Probes) or streptavidin-fluorescein isothiocyanate (FITC) (Molecular Probes) diluted 1:200 in PBS/1% BSA and incubated on ice for 30 min. DNA was stained by incubating cells with Hoescht 33342 (Molecular Probes) at 10 μg/ml on ice for 30 min. Cells were washed twice and analyzed by flow cytometry. Dead cells were stained with 1 μg/ml propidium iodide (Sigma).

Total light-density fetal liver cells and GPA^- light-density fetal liver cells were analyzed for binding of FITC-labeled phage and PE-labeled GPA or CD34 (Beckman Coulter), respectively. Binding of FITC-labeled phage was directly compared with binding of FITC-labeled CD36 (Beckman Coulter) and CD71 (Becton Dickinson Immunchemistry Systems) mAbs. Phage were directly labeled with FITC by incubating on ice, for 90 min, 10^{12} phage in 500 μl of 100 mM $NaHCO_3$ (pH 8.5) with 5 μl of 6-[fluorescein-5-(and -6)-carboxamido]hexanoic acid, succinimidyl ester [5(6)-SFX] (Molecular Probes) suspended at 10 mg/ml in *N,N*-dimethyl formamide. Phage was precipitated with PEG and resuspended three times. After labeling with FITC, the phage were resuspended in 500 μl of PBS/1% BSA and then used for cell staining. Approximately $1\text{–}5 \times 10^5$ cells were stained, in a volume of 20–50 μl in 96-well V-bottom plates (Costar), with saturating levels of labeled phage or mAbs for 30 min on ice. Thereafter, the cells were washed twice with washing buffer consisting of PBS containing 0.5% BSA and 0.01% NaN_3. The cells were resuspended in the same washing buffer supplemented with propidium iodide for the analysis of live cells.

Immunohistochemisty and Immunofluorescence Microscopy. For cell staining, biotinylated phage was used. Phage was biotinylated by incubating 10^{12} phage in 500 μl of 100 mM $NaHCO_3$ (pH 8.5) with 40 μl of 2 mg/ml Sulfo-NHS-LC-biotin (Pierce) for 30 min on ice. Phage was precipitated with PEG and resuspended three times. For staining, 10^6 cells were first blocked in a total volume of 100 μl of PBS/1% BSA containing the helper phage M13K07 at a concentration of 10^{13}/ml for 20 min. Ten microliters of biotinylated phage antibody was added to the cells and incubated for 30 min on ice. Cells were washed with PBS/1% BSA and used to prepare slides for immunohistochemistry and immunofluorescence using a cytocentrifuge (Sakura Finetek, Torrance, CA). Slides were air dried overnight and fixed with 2% formaldehyde at room temperature for 20 min. After washing, phage staining was detected by alkaline phosphatase conjugated streptavidin (DAKO) diluted 1:120 with PBS at room temperature for 20 min. Slides were washed twice in PBS and then developed with Fast Red (DAKO) at room temperature for 20 min. For immunofluorescence, phage binding was detected by incubation with Alexa fluor 546-conjugated streptavidin (Molecular Probes) diluted 1:400 with PBS. Fetal hemoglobin was

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also detected by incubation with anti-γ (Hb F) mAb (Perkin-Elmer Wallace) diluted 1:400, followed by staining with a goat anti-mouse FITC-conjugated antibody (Caltag) (18). DNA was stained with 4′,6-diamidino-2-phenylindole mounting media (Vector Laboratories). The stained cells were evaluated by using Zeiss Axiophot fluorescence microscope with filters (UV, blue, or green excitation) for 4′,6-diamidino-2-phenylindole, FITC, or Alexa fluor 546, respectively, and a dual band filter (blue and green excitation) for combined FITC and Alexa 546 detection.

Results

To generate mAbs to fetal erythroid antigens, a scFv phage antibody library was constructed in the phage vector fdDOG (6). Unlike antibody libraries constructed in phagemid vectors, use of a phage vector yields three to five copies of scFv-pIII fusion per phage, resulting in multivalent display. Such multivalent display confers an increase in the functional affinity constant of the phage antibodies and should theoretically result in both more efficient depletion of antibodies to antigens in common with adult cells, and more efficient positive selection of antibodies to fetal antigens. To construct a nonimmune phage antibody library, an scFv gene repertoire was subcloned as *Sfi*I–*Not*I gene fragments from a large nonimmune phage antibody library in the phagemid vector pHEN1 into the phage vector fd. After transformation, a library of 3.2×10^8 tetracycline-resistant clones was obtained. PCR screening revealed that 95% of clones had an scFv size insert, yielding a functional library size of 3.04×10^8 members. To verify that scFv display levels were higher in phage vs. phagemid vectors, phage were prepared from both libraries, and subjected to SDS/PAGE followed by Western blotting with an anti-pIII antibody. Blots indicated that for the phage library, approximately 50% of pIII consisted of scFv fusion. In contrast, only 1–5% of pIII consisted of scFv fusion for the phagemid library (data not shown).

To generate a panel of mAbs to fetal NRBC antigens, the phage library was panned on fetal nucleated erythroid cells obtained from fetal liver. Because fetal erythroid cells express antigens that are also present on some adult WBCs and RBCs, the phage library was first extensively depleted against both adult RBCs and adult WBCs. After the first round of selection, the titer of the recovered phage was 3×10^4. The recovered phage were amplified by growth in bacteria and used for a second round of selection. After the second round, the titer of the recovered phage was 5×10^5.

To determine the outcome of the selection strategy, polyclonal phage were prepared after the second round of selection and analyzed for binding to adult buffy coat WBCs and total fetal liver cells by flow cytometry. Only a very small amount of binding was detected on adult WBCs, whereas the majority of cells from the total fetal liver bound phage (Fig. 1). To identify phage that specifically bound fetal erythroid antigens, phage were prepared from individual colonies from the second round of selection and analyzed for fetal erythroid cell binding and adult WBC binding by flow cytometry. Of 95 random clones analyzed, 42 bound fetal erythroid cells by flow cytometry. Two-thirds of the antibodies showed no evidence of WBC binding (for example, mAb fd-H7 in Fig. 2). One-third of the mAbs bound a small percentage of WBC from the buffy coat as determined by flow cytometry (for example, mAb fd-B6 in Fig. 2). To determine the number of unique antibodies generated, the scFv gene was amplified by PCR and the PCR product was digested with the frequently cutting restriction enzyme *Bst*N1 (PCR fingerprinting). From the 42 fetal erythroid binding scFv, 16 unique fingerprints were observed, indicating the presence of 16 unique antibodies (Fig. 3).

These 16 phage antibodies were analyzed according to their binding to fetal liver derived light-density cells that are comprised predominantly of NRBCs but also of hematopoietic

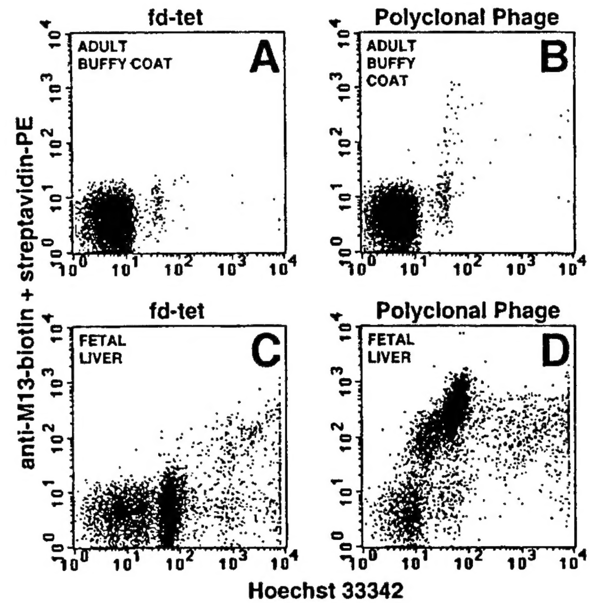

Fig. 1. Binding of polyclonal phage antibodies to adult buffy coat WBCs and total fetal liver cells. After the second round of selection, polyclonal phage were prepared and analyzed for their ability to bind either adult buffy coat WBCs (*B*) or total fetal liver (*D*). There is a large shift in the fetal liver cells compared with a minimal shift of a small population of adult buffy coat WBCs. In contrast, control wild-type fd phage did not significantly shift either cell population (*A* and *C*).

progenitors as well as mature leukocytes (Table 1). The phage antibodies bound 70–99% of the GPA^+ population of these light-density cells. The binding to the GPA^- population was more variable, ranging from 7.5–71.5% cells, suggesting that they were binding to different antigens on the more primitive erythroid cells. These phage antibodies were then further analyzed for their binding to $CD34^+$ or $CD34^-$ cells. Their binding properties were also compared with those of anti-CD36 and anti-CD71 mAbs. By these criteria, as well as by judging the FACS analysis patterns, the phage antibodies could be grouped into five clusters. Two of them that had the binding characteristics similar to antibodies against CD36 or CD71 also demonstrated appreciable binding to adult peripheral blood cells. The other three clusters, A, B, and C, bound fetal liver-derived, GPA^+ light-density cells but not mature RBCs. They differ from one another in that clusters A and C bound more GPA^+ cells than cluster B did, and cluster C bound more $CD34^+$ cells from the GPA^- fraction than clusters A and B did. The antigen detected by cluster B also appeared to be more restricted to GPA^+ cells. These results indicate that the phage antibodies that we have generated are binding to different antigens on immature erythroid and hematopoietic cells other than CD36 and CD71.

To confirm further which of these antibodies were binding specifically to fetal NRBCs, immunohistochemistry was performed on total fetal liver and on adult WBC buffy coats. About two-thirds of the antibodies stained only the erythroid cells in fetal liver but not the buffy coat WBCs (Fig. 4 *A* and *C*). The other one-third stained erythroid cells in fetal liver and also adult monocytes (Fig. 4 *B* and *D*). These results confirm the specificity demonstrated by flow cytometry. As an additional demonstration of specificity, immunofluorescence was performed on fetal liver cells, and a representative result is shown in Fig. 5. Only fetal hemoglobin containing cells with nuclei (Fig. 5*D*) were

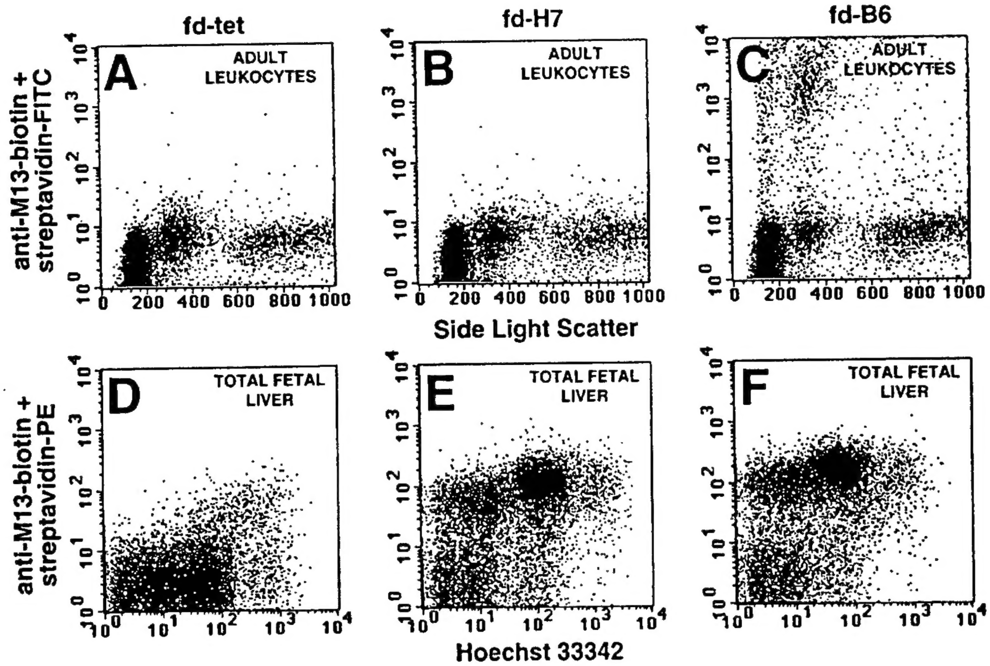

Fig. 2. Binding of monoclonal phage antibodies to adult buffy coat WBCs and total fetal liver cells. Phage were prepared from individual colonies after the second round of selection and analyzed for their ability to bind adult buffy coat WBCs or total fetal liver cells. The representative antibody fd-H7 (*B* and *E*) bound only fetal liver cells, whereas the antibody fd-B6 (*C* and *F*) bound fetal liver and a small subpopulation of WBCs consistent with monocytes. Staining with wild-type fd phage (*A* and *D*) is included as a control.

stained by the FITC-tagged anti-fetal hemoglobin mAb (Fig. 5*A*) and by the Alexa 546-tagged phage antibody (Fig. 5*B*). When visualized with a dual band filter, the fetal NRBCs yielded yellow fluorescence (Fig. 5*C*). The two enucleated fetal RBCs stained only green with FITC, whereas the WBC stained with neither.

Discussion

Production of mAbs by xenotypic immunization of mice with intact human cells usually results in the production of antibodies against immunodominant epitopes found on more than one cell type. As a result, it is impossible to generate a complete set of antibodies to surface receptors by using hybridoma technology. Phage antibody libraries represent a potential solution to this problem, and successful cell surface selections have been reported by using fixed (19) or living cells (12, 13, 20–23). However, because of the heterogeneity of the starting material, such selections require elaborate subtraction protocols to avoid the selection of irrelevant antibodies. Although there are several reports of successful selections on cells using large nonimmune libraries (10, 12, 13), this approach has been most successful by using small libraries from immunized sources. This limits the spectrum of antibody specificities obtainable from any single library to those present on the immunizing cell, and does not completely overcome the problem of immunodominant epitopes. Furthermore, the antibodies obtained are usually murine.

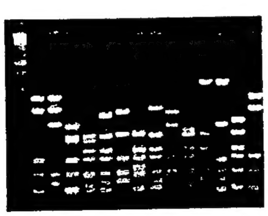

Fig. 3. DNA fingerprint analysis of the scFv genes of individual antibodies from the second round of selection. scFv DNA was amplified by PCR directly from colonies and digested with the frequently cutting restriction enzyme *Bst*N1. A diverse banding pattern was observed, with each unique pattern representing a unique antibody sequence. First lane is a 100-bp DNA marker.

Table 1. Binding of phage antibodies by subsets of fetal liver cells

Antibody fragment/mAb*	Light-density fetal liver		GPA^- light-density fetal liver	
	GPA^+	GPA^-	$CD34^+$	$CD34^-$
Cluster A				
FSH8	90.9%	16.9%	6.6%	5.3%
FSH7	92.1%	39.6%	2.0%	3.9%
FSG5	98.7%	66.7%	7.0%	6.7%
FSG9	86.2%	45.7%	3.1%	4.2%
Cluster B				
C7	71.6%	7.5%	5.5%	4.8%
F4	81.7%	9.8%	5.4%	4.6%
C3	84.1%	7.9%	7.1%	6.5%
B5	88.0%	8.0%	9.9%	8.7%
Cluster C				
FSE2	99.2%	46.9%	18.4%	8.2%
A6	81.6%	11.3%	14.8%	6.2%
CD36*	98.4%	34.6%	43.2%	25.7%
FSH3	90.7%	22.0%	22.9%	18.9%
FSD8	99.2%	53.6%	53.7%	51.5%
CD71*	99.2%	47.8%	52.0%	13.7%
FSA7	97.9%	30.3%	42.8%	20.0%
FSD10	99.4%	51.3%	45.0%	18.0%
H7	99.3%	71.5%	61.9%	23.0%
G4	99.1%	34.0%	39.0%	13.8%

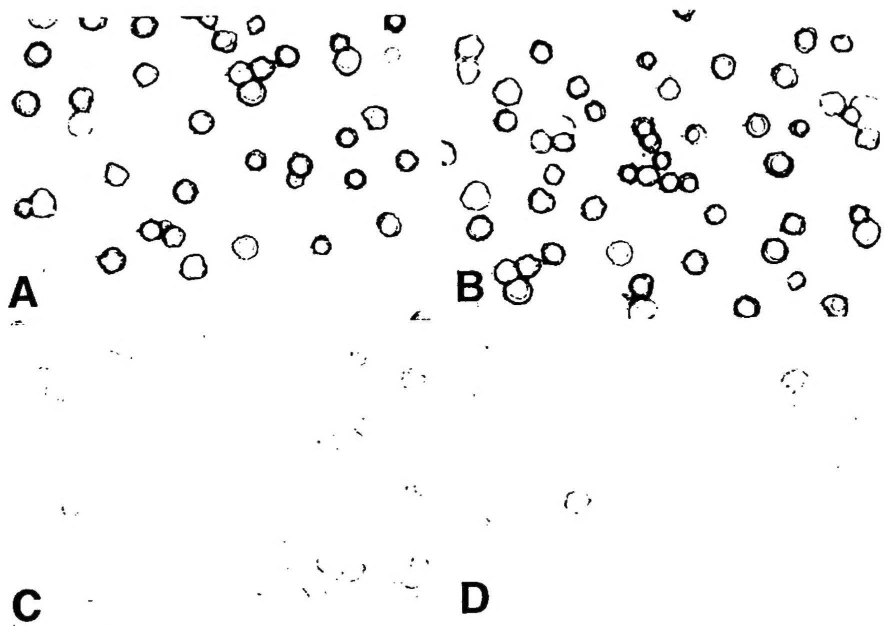

Fig. 4. Staining of adult buffy coat WBCs and total fetal liver by phage antibodies. Adult buffy coat WBCs or fetal liver cells were stained with biotinylated phage antibodies and the cells applied to microscope slides, fixed, and stained with streptavidin-alkaline phosphatase and Fast Red. The phage antibody FSA7 stains fetal erythroid cells (*A*) but not WBCs (*C*), whereas the phage antibody FSH3 stains both fetal liver (*B*) and a subset of WBCs (*D*).

The step limiting the selection of binders from large naïve libraries by cell panning seems to be the relatively high background binding of nonspecific phage and relatively low binding of specific phage (24–26). The low binding of specific phage is partially related to the low concentration of a given binding phage in the polyclonal preparation (approximately 1.6×10^{-17} M for a single member of a 10^9 library in a phage preparation of 1×10^{13} particles per ml). The low concentration simultaneously limits the efficiency of both subtraction of common binders and enrichment of specific binders. To overcome this limitation, we

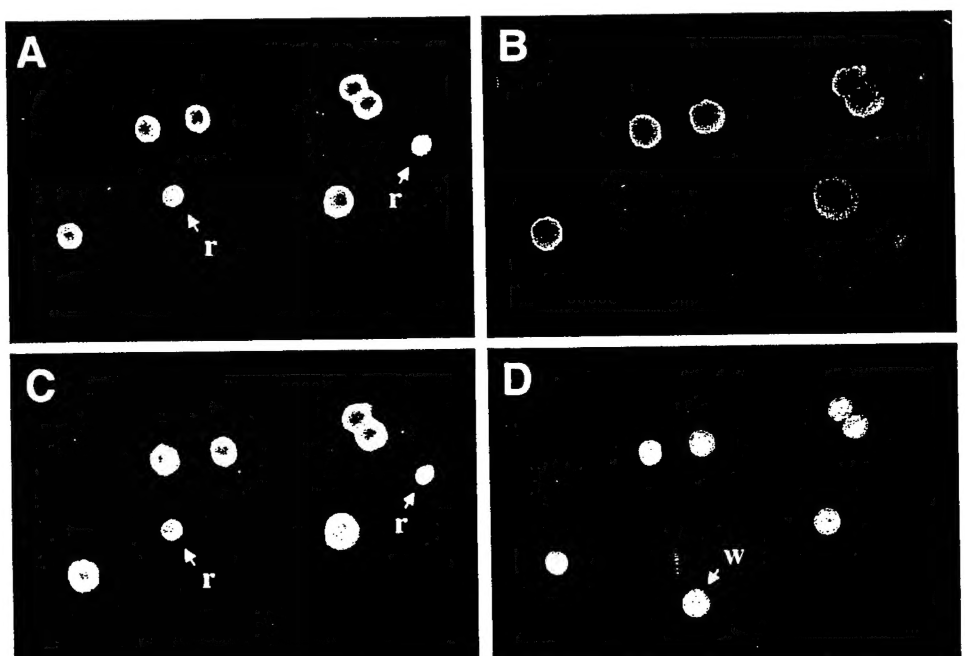

Fig. 5. Staining of total fetal liver by a fetal NRBC specific phage antibody. Fetal liver cells were incubated with biotinylated phage antibody FSG9, applied to microscope slides, and then stained with FITC anti-fetal hemoglobin antibody (*A*), streptavidin Alexa fluor 546 to detect phage binding (*B*), or 4′,6-diamidino-2-phenylindole (*D*). Fetal hemoglobin and phage binding were visualized together with a dual band filter (*C*). Only fetal NRBCs stained with both. r, enucleated RBC; w, WBC.

generated a large nonimmune phage library in the phage vector fd. Compared with existing nonimmune libraries constructed in phagemid vectors, such phage vectors will display an antibody fragment on each of the five copies of pIII, leading to multivalent antibody display. Multivalent display leads to an increase in the functional affinity constant of the phage antibody, resulting in both increased efficiency of depletion and positive selection. In contrast, phagemid libraries display less than one antibody fragment per phage because of the presence of wild-type pIII from the helper phage.

For the purposes of prenatal genetic diagnosis, there are several advantages of targeting fetal erythroid cells. Fetal NRBCs are already found in the mother's blood during the first trimester and are present throughout the entire pregnancy. They have a limited lifespan and do not persist from prior pregnancies as it has been found with fetal lymphocytes (4). Although fetal NRBCs are an ideal source of fetal genetic material, they are present at an extremely low frequency in the absence of fetomaternal hemorrhage. It is estimated that the frequency is one fetal NRBC per 10^6–10^8 maternal nucleated cells (4). Hence, antibodies specific for fetal cells will be invaluable for their enrichment and confirmation.

At present, antibodies to fetal and embryonic hemoglobin are being used to identify fetal RBCs. However, Hb F in the maternal RBC may be elevated in some pregnancies, and although anti-ζ antibodies are more specific, embryonic hemoglobin has a narrower temporal window of expression (27). Additionally, targeting intracellular antigens makes the purification steps more subject to cell loss, as the fragile erythroid cells need to be permeabilized to make the hemoglobin accessible to these antibodies. Thus, cell surface markers are preferred for enrichment. Cell surface antibodies to CD71, CD36, and I/i antigens are most commonly used for purification. However, they have the disadvantage of being expressed on subpopulations of maternal WBCs. Because of this fact, additional erythroid markers are likely to be useful for fetal cell enrichment or verification of fetal RBCs. Preliminary results of phage antibodies generated by panning on fetal liver cells have also been reported by others (27, 28). The panel of antibodies we have generated are likely to span a wide range of fetal RBC antigens other than CD71, CD36, and I/i. Further characterization of the specificity and utility of these phage antibodies for erythroid and hematopoietic cells of different lineage in the normal and disease states is in progress. If, for example some can be found to be specific for primitive erythroid cells, they may prove useful not only for prenatal diagnosis, but also for treatment of polycythemia or erythroleukemia.

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